Sarcopenie : mécanismes et prévention : rôle de l'exercice et de l'hormone de croissance : implication du stress oxydant et de la glucose-6-phosphate déshydrogénase

Thomas Brioche

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Thomas Brioche

Préparée au sein des laboratoires Free Radical and Antioxidant Research Group (Université de Valencia, Espagne) et Mouvement, Sport, Santé (Université Rennes 2 – ENS Rennes, France)

Sarcopenia: Mechanisms and Prevention
Role of Exercise and Growth Hormone

Involvement of oxidative stress and Glucose-6-phosphate dehydrogenase

Thèse soutenue le 09 Avril 2014
devant le jury composé de :

Angèle CHOPARD
Professeur - UMR 866 DMEM, Montpellier / rapporteur

Damien FREYSSENET
Professeur - Laboratoire de Physiologie de l'Exercice, Saint-Etienne / rapporteur

Isabelle PETROPOULOS
Professeur - Laboratoire de Biologie Cellulaire du Vieillissement, Paris / Examinateur

José VINA
Professeur - Laboratoire FRAG, directeur de thèse

Arlette GRATAS-DELAMARCHE
Professeur - Laboratoire M2S, directrice de thèse

Mari-Carmen GOMEZ-CABRERA
Professeur - Laboratoire FRAG, Co-directrice de thèse

Sophie LEMOINE-MOREL
Maître de conférences - Laboratoire M2S, Co-directrice de thèse

What's doesn't kill you makes you stronger…

Friedrich Nietzsche

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Abbreviations

3-NT	3-nytrotyrosine	**Cat**	Catalase
4E-BP1	Eukaryotic initiation factor 4E binding protein 1	**CKI**	Cyclin-dependent kinases
4-HNE	4-hydroxynonenal	**CS**	Citrate synthase
8-OHdG	8-oxo-deoxyguanosine	**CSA**	Cross sectional area
8-OHG	8-oxo-oxyguanosine	**COX I**	Cytochrome c oxidase I
γ-GCLC	γ-glutamate-cysteine ligase	**CT**	Computed tomography
AAS	Availability of amino acids	**CuZn-SOD**	Copper-Zinc Super oxide dismutase
ADP	Adenosine disphosphate	**CyPD**	Cyclophilin D
AGS	Automatic Grip Strength	**DHEA**	Dehydroepiandrosterone Sulphate
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside	**DNA**	Deoxyribonucleic acid
AIF	Apoptosis-Inducing Factor	**Drp 1**	Dynamin-related protein 1
Akt	Protein kinase B	**DXA**	Dual-energy x-ray absorptiometry
AMP	Adenosine monophosphate	**EDL**	Extensor digitorum longus
AMPK	AMP-activated protein Kinase	**eEF2**	Eukaryotic elongation factor-2
ANT	Adenine nucleotide translocator	**eEF2K**	Eukaryotic elongation factor-2 kinase
Apaf-1	Apoptosis Protease-activating factor 1	**EGCG**	Epigallocatechin-3-gallate supplementation
ASM	Appendicular skeletal muscle mass	**eIF2B**	Eukaryotic translation initiation factor 2B
ATF2	Activating transcription factor 2	**eIF3-f**	Eukaryotic translation initiation factor 3-subunit F
Atg	Atrogin	**eIF4B**	Eukaryotic translation initiation factor 4B
ATP	Adenosine triphosphate	**eIF4E**	Eukaryotic translation initiation factor 4E
ATPase	Adenosine triphosphatase	**EndoG**	Endonuclease G
Bad	Bcl-2-associated death promoter	**eNOS**	Endothelial nitric oxide synthase
Bak	Bcl-2 homologous antagonist/killer	**EPSESE**	Established Populations of Epidemiologic Studies of the Elderly
Bax	Bcl-2–associated X protein	**ERK1/2**	Extracellular signal Regulated Kinase 1/2
Bcl-2	B-cell lymphoma 2	**ESPEN**	European Society of Clinical Nutrition and Metabolism
Bcl-XL	B-cell lymphoma-extra large	**ETC**	Electron transport chain
BIA	Bioelectric impedance	**EWGSOP**	European Working Group on Sarcopenia in Older People
BMD	Bone mineral density	**FAD**	Flavine adenine dinucleotide

Bnip3	BCL2/adenovirus protein-interacting protein3	**Fis 1**	Fission 1 homolog
Bnip3L	BCL2/adenovirus protein-interacting protein3-like	**MRFs**	Myogenic regulatory factors
FoxO	Forkhead box O	**MRI**	Magnetic resonance imaging
G6PDH	Glucose-6-phosphate dehydrogenase	**mRNA**	messenger RNA
GDH	Glutamate dehydrogenase	**Mstn**	Myostatin
GH	Growth hormone	**mtDNA**	Mitochondrial DNA
GHRH	Growth hormone-releasing hormone	**mTOR**	Mammalian target of rapamycin
Gpx	Glutathione peroxidase	**mTORC1 and 2**	Mammalian target of rapamycin complex 1 and 2
GR	Glutathione reductase	**MuRF1**	Muscle-specific RING-finger protein 1
GSH	Reducted glutathione	**MHC**	Myosin heavy chain
GSK-3	Glycogen Synthase Kinase 3	**N**	Nitrogen
GSSG	Oxidized glutathione	**NAD**	Nicotinamide adenine dinucleotide
GTPases	Guanosine triphosphatases	**NADPH**	Nicotinamide adenine dinucleotide phosphate
H2O2	Hydrogen peroxide	**NAMPT**	Nicotinamide phosphoribosyltransferase
HNA	4-hydroxy-2-nonenoic acid	**nDNA**	Nuclear DNA
$HO^{\bullet}$	Hydroxyl radical	**NFAT**	Nuclear factor of activated T-cells
HPLC	High-performance liquid chromatography	**NFκB**	Nuclear Factor Kappa B
HSPs	Heat shock proteins	**nNOS**	Neuronal nitric oxide synthase
ICDH	Isocitrate dehydrogenase	**NO**	$NO^{\bullet}$ Nitric oxide
IGF-1	Insulin-like Growth Factor I	**NOS**	Nitric Oxide synthase
IGF1R	Insulin-like Growth Factor I Receptor	**NOX**	NADPH oxidase
IL-6	Interleukin-6	**NRF-1 and 2**	Nuclear respiratory factor 1 and 2
IM	Inner membrane	**O_2**	Oxygen
IMS	Intermembrane space	**$O_2^{-\bullet}$**	Superoxide radical
IRS1	Insulin receptor substrate 1	**OGG1**	Oxoguanine DNA glycosylase
IWGS	International Working Group on Sarcopenia	**$OH^{\bullet}$**	Hydroxyl radical
KO	Knot out	**$ONOO^{\bullet}$**	Peroxynitrite anion
MAFbx	Atrogin-1	**Opa 1**	Optic atrophy1
MAP kinase	Mitogen-activated protein kinase	**OS**	Oxidative stress
MDA	Malondialdehyde	**OXPHOS**	Oxidative phosphorylation
ME	Malic enzyme	**p70S6K**	70-kDa ribosomal protein S6 kinase
MEF2	Myocyte enhancer factor-2	**PCR**	Polymerase Chain Reaction
Mn-SOD	Manganese Super oxide dismutase	**PDK-1**	Phosphoinositide-dependent kinase-1

mPTP	Mitochondrial permeability transition pore	**SOD**	Superoxide dismutase
PGC-1α	PPAR gamma coactivator 1 alpha	**SPPB**	Short Physical Performance Battery
PGC1-β	PPAR gamma coactivator 1beta	**SSCWD**	Society of Sarcopenia, Cachexia and Wasting Disorders
PGD	6-phosphogluconate dehydrogenase	**STAT3**	Signal Transducer and Activator of Transcription 3
PI3K	Phosphatidylinositol-3-kinase	**TBARS**	Thiobarbituric acid reactive
PINK1	PTEN-induced putative kinase 1	**TFAM**	Mitochondrial transcription factor A
PIP2	Phosphoinositide-(4,5)-biphosphate	**TGF-β**	Transforming Growth Factor beta
PIP3	Phosphoinositide-(3,4,5)-triphosphate	**TNF-α**	Tumor Necrosis Factor α
PLA2	Phospholipase A2	**Trx**	Thioredoxin
PPARα	Peroxisome proliferator-activated receptors α	**TSC1**	Tuberous Sceloris protein 1
PPP	Pentose phosphate pathway	**TSC2**	Tuberous Sceloris protein 2
PUFA	Polyunsaturated fatty acids	**TUG**	Timed Get-up-and-go
R5P	Ribose-5-phosphate	**TWEAK**	TNF-like weak inducer of apoptosis
REDD1 and 2	Regulated in Development and DNA damage responses 1 and 2	**Ulk1**	Unc-51-like kinase 1
RGPE	Red grape polyphenol extract	**UPS**	Ubiquitin-proteasome system
RNA	Ribonucleic acid	**VDAC**	Voltage-dependent anion channel
RNS	Reactive species derived from of nitrogen	**VO_2max**	Maximal oxygen uptake
ROO$^{\bullet}$	Peroxyl radical	**Wa**	Animal's carcass weight in the air
ROOH	Lipid hydroperoxide	**WT**	Wild type
RONS	Reactive species derived from of oxygen and nitrogen	**Ww**	Animal's carcass weight in the water
ROS	Reactive species derived from of oxygen	**XDH**	Xanthine dehydrogenase
rpS6	Ribosomal protein S6	**XO**	Xanthine oxidase
RyR1	Ryanodine receptor 1	**XOR**	Xanthine oxidoreductase
SC	Satellite cells		
SCPT	Stair climb power test		
SD	Standard deviations		
SDH	Succinate dehydrogenase		
SERCA2	Sarco/endoplasmic reticulum Ca2+-ATPase		
SGI	Specific Gravity Index		
-SH	Thiols residues		
SIRT	Sirtuin		
SMI	Skeletal muscle mass index		

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INTRODUCTION

Introduction

Around eighty years ago, MacDonald Critchley was the first to recognize that muscle mass decreases with aging and noted that it is most noticeable in intrinsic hand and foot muscles (Critchley 1931). Almost sixty years later, in 1988, during a meeting convened in Albuquerque (USA) which provided information and updated the assessment of health and nutrition in older populations, Rosenberg, noted that 'no decline with age is more dramatic or potentially more functionally significant than the decline in muscle mass'. He highlighted the interest that to provide recognition by the scientific community, this phenomenon needed a name and proposed the term 'sarcopenia' (Greek *'sarx'* or flesh + *'penia'* or loss). Thereafter, sarcopenia was defined as the progressive general decline in muscle mass that occurs with aging (Roubenoff & Hughes 2000). However, this definition was not accepted by all the clinicians and investigators and has been evolved a lot until few years. Finally, the actual consensus defines sarcopenia as 'a geriatric syndrome initially characterized by a decrease in muscle mass that will get worse causing a deterioration in strength and physical performance' (Muscaritoli et al. 2010; Cruz-Jentoft et al. 2010; Fielding et al. 2011; Morley et al. 2011).

Due to social, technological and medical progress, the life expectancy has been increasing since the 19th century in our modern Western societies, leading to the aging of the general population. Currently, it is projected that the number of elderly will double worldwide from 11% of the population to 22% by 2050 (UN 2007). Inevitably, due to this aging population, prevalence of sarcopenia is growing, and currently it is estimated that one-quarter to one-half of men and women aged 65 and older are likely sarcopenic (Janssen 2004). The consequences of the increasing prevalence of sarcopenia are generally considered as catastrophic on the public health costs. Thus, the total cost of sarcopenia to the American Health System has been reported to be approximately $18.4 billion (Janssen et al. 2004). This cost would worse in the future since individuals over the age of 69 years are the largest growing segment of the American population (Manton and Vaupel 1995). These healthcare costs are linked to a general deterioration of the physical condition resulting in an increased risk of falls, a progressive inability to perform basic activities of the daily life and loss of independence of the elderly (Goodpaster et al 2006, Delmonico et al 2007).

However, several strategies are acknowledged as effective to prevent, delay, or treat age-related sarcopenia. Thus, developing therapies will not only help to enhance the quality of life for individual sarcopenic patients but also reduce the economic and productivity burdens associated with sarcopenia, and would be beneficial to society as a whole. Exercise training is surely the most effective in counteracting sarcopenia since it can lead to increase muscle mass, strength and physical performance (Pillard et al. 2011; Di Luigi et al. 2012; Wang &

Bai 2012; Montero & Serra 2013). However, the large scale implementation of such intervention is hampered by the lack of motivation of most persons. In addition, many elderlies are non-ambulatory or have co-morbidities such as moderate to severe osteoarthritis (Bennell & Hinman 2011) or certain forms of unstable cardiovascular disease that would preclude participation in resistance training exercises (Williams et al. 2007). To overcome such barriers, developing alternative therapies such as antioxidant strategies and hormone replacement therapies (testosterone and GH) appear to be necessary.

Skeletal muscle is an organ which has specific properties that give it a central role in locomotion, performing activities of the daily life and the maintenance of posture and balance. In order to ensure these essential functions, it must have a sufficient mass and seek to preserve it. As previously described, some of the most serious consequences of ageing are its effects on skeletal muscle particularly the progressive loss of mass and function which impacts on quality of life, and ultimately on survival (Cruz-Jentoft 2012). The underlying mechanisms of sarcopenia are still under investigation. However, a negative protein turnover (Combaret et al. 2009), impaired mitochondrial dynamics (Calvani et al. 2013), a decreased muscle regeneration capacity (Snijders et al. 2009; Hikida 2011), as well as an exacerbation of apoptosis (Marzetti et al. 2012) are usually considered to be cellular mechanisms involved in muscle atrophy leading to sarcopenia.

These mechanisms are themselves dependent on a multitude of systemic and cellular factors such as decreased production of anabolic hormones (GH, IGF-1, testosterone, insulin). Links and interactions between these depleted hormones and the cellular dysfunctions previously cited remain partly unknown. A potential candidate could be the age-related chronic oxidative stress, whose recent studies emphasized its involvement in sarcopenia (Semba et al. 2007; Safdar et al. 2010). Thus, sarcopenic muscle exhibits increased free radicals derived from oxygen and nitrogen (RONS) production (Capel et al. 2004; Capel, Rimbert, et al. 2005; Capel, Demaison, et al. 2005; Chabi et al. 2008; Jackson et al. 2011; Andersson et al. 2011; Miller et al. 2012). This overproduction of RONS is mainly due to mitochondrial dysfunctions (Capel, Rimbert, et al. 2005; Chabi et al. 2008) and increased xanthine oxidase activity (Lambertucci et al. 2007; Ryan et al. 2011), and leads to an increase in oxidative damage to skeletal muscle cellular components. These oxidative damage reflect the inability of antioxidant systems to contain this RONS overproduction and attests an imbalance of the "oxidants-antioxidants" balance leading to an impaired redox homeostasis. It seems that the restoration of redox homeostasis by the different preventive strategies previously exposed involves an up-regulation of the glucose-6-phosphate dehydrogenase

(G6PDH) enzyme muscle protein content and/or activity (Kovacheva et al. 2010; Sinha-Hikim et al. 2013). G6PDH is the first and rate-limiting enzyme of the pentose phosphate pathway which would supply NADPH to several antioxidant systems (M. D. Scott et al. 1993). Moreover, few data *in vitro* or *in vivo* have suggested that G6PDH would play an important role in muscle mass regulation. However, these data need to be confirmed.

In this context, this thesis will attempt to answer three general objectives. The first objective is to determine *in vivo* to what extent a pro-oxidant redox status within the aged muscle tissue may modulate signaling pathways involved in cellular mechanisms underlying sarcopenia. The second objective is to show that return to normal functioning of these signaling pathways requires a restoration the redox homeostasis. Finally, the third objective of this thesis is to identify actors and their possible cellular mechanisms in the maintenance and/or the restoration of the redox status.

REVIEW

Chapter 1: What is Sarcopenia?

1. Definitions of sarcopenia

1.1. The origins of the word "Sarcopenia"

A reduction in lean body mass and an increase in fat mass is one of the most striking and consistent changes associated with advancing age. Skeletal muscle and bone mass are the principal components of lean body mass to decline with age (Tzankoff & Norris 1978). These changes in body composition appear to occur throughout life and have important functional and metabolic consequences. In 1931, MacDonald Critchley was the first to recognize that muscle mass decreases with aging and noted that it is most noticeable in intrinsic hand and foot muscles (Critchley 1931). At the beginning of the 1970's, Forbes was the first researcher to report prospective data on the age-related decrease in muscle mass in a small group of adults using potassium^{40} counting data (Forbes & Reina 1970). The reported decline was -0.41% per year as obtained in 13 men and women aged between 22 and 48 years old. Evidence suggests that up to 40% of muscle mass may be lost between the ages of 20 and 70 years (Rogers & Evans 1993) and can exceed over 50% among those aged 80 years and older (Baumgartner et al. 1998). The decline of skeletal muscle mass may accelerate along with aging, which is 6% per decade between 30 and 70 years of age (Fleg & Lakatta 1988), 1.4% to 2.5% per year after age 60, and could start as early at 35 years of age (Frontera & Hughes 2000).

In 1988, Irwin Rosenberg noted that 'no decline with age is more dramatic or potentially more functionally significant than the decline in muscle mass' and proposed for the first time, the term 'sarcopenia' (Greek 'sarx' or flesh + 'penia' or loss) to describe this age-related decrease of muscle mass (Rosenberg 1989).

1.2. First definitions based only on muscle mass

So, sarcopenia was first defined as the progressive general decline in muscle mass that occurs with aging (Roubenoff & Hughes 2000).

The first epidemiological studies fixed to a strict definition of sarcopenia as loss of muscle mass. In this context, some studies have suggested criteria based on the use of dual-energy x-ray absorptiometry (DXA) to quantify muscle mass. For instance, Baumgartner et al. (1998) summed the muscle mass of the four limbs as appendicular skeletal muscle mass

(ASM), and expressed muscle mass as ASM/height² (as kg/m²). Individuals with a ASM/height² two standard deviations (SD) below the mean of a middle-age reference male and female population (aged 18-40 years) from the Rosetta study (Gallagher et al. 1997) were defined as gender-specific cutpoints for sarcopenia. Later, others proposed the use of a skeletal muscle mass index (SMI) based on the total skeletal muscle mass divided by the body weight and multiplied by 100 (Janssen et al. 2002). With this definition, two stages of sarcopenia are considered: a stage 1 when the index is between 1 and 2 standard deviations compared to a younger population of reference, a stage 2 when the index is less than 2 standard deviations (Janssen et al. 2002). Another method based on appendicular skeletal muscle mass adjusted for height and body fat mass (also called residuals) was proposed by Newman et al. in 2003 and showed that fat mass should be considered in estimating prevalence of sarcopenia in women and in overweight or obese individuals (Newman et al. 2003). This method began to show some limits of a definition based only on muscle mass.

1.3. Limits of only using muscle mass to define sarcopenia

There are many crucial aspects of sarcopenia that are missed by the unique use of muscle mass. Relevant patient outcomes of sarcopenia include mortality and physical disability (i.e. the inability to walk or perform activities of daily living). Some studies have shown that reduced skeletal muscle mass is predictive of disability and mortality but numerous studies have shown that muscle mass by itself is a weak predictor of outcomes (Visser et al. 2000; Visser et al. 2005; Newman et al. 2006; Gale et al. 2007; Hairi et al. 2010; Goodpaster et al. 2006). It has also been shown that the relation between muscle mass, muscle function (strength and power) is not linear (Goodpaster et al. 2006; Janssen 2004). Indeed, although loss of strength tends to track with loss of muscle mass with aging without any pathologies, the decline in muscle strength is steeper than the decline in muscle mass (Frontera & Hughes 2000; Doherty 2003). Moreover, interventions that increase muscle mass do not necessarily increase muscle strength (Wittert et al. 2003). Furthermore, changes in muscle strength that occur with resistance training precede measurable changes in muscle mass temporally and exceed them in size (Sillanpää et al. 2009). On the other hand, loss in strength is not necessarily present with voluntary weight loss despite the associated loss of skeletal muscle (Wang et al. 2007). Finally, correlations between change in muscle mass and change in strength in older adults are inconsistent and not very robust (Goodpaster et al. 2006).

Some reasons can explain this dichotomy between muscle mass and strength such as age-related infiltration into skeletal muscle by fat, which is a powerful predictor of future disability and mortality (Visser et al. 2005).

Finally, the limit of only using skeletal muscle mass to define sarcopenia is the variety of measures available to evaluate this compartment. Each of these leads to slightly different cutoffs for muscle mass and are indirect measures. As such, they can be influenced by adiposity and total body water (Dumler n.d.; Heyward 1996; Omran & Morley 2000). These different methods (DXA, Computed Tomography, Magnetic Resonance Imagery, and Bioelectrical Impedance) will be presented in another chapter.

Given the inconsistency of the sarcopenia definition based only on muscle mass, and the evidence that this latest has practical limitations, since 2005 several groups from the United States and Europe have redefined sarcopenia.

1.4. Consensus definitions of sarcopenia

Four working groups (the European Society of Clinical Nutrition and Metabolism: ESPEN; the European Working Group on Sarcopenia in Older People: EWGSOP; the International Working Group on Sarcopenia: IWGS; the Society of Sarcopenia, Cachexia and Wasting Disorders: SSCWD) published recently international consensus definitions (Muscaritoli et al. 2010; Cruz-Jentoft et al. 2010; Fielding et al. 2011; Morley et al. 2011) that will be presented in chronological order of publication. Other study groups, such as the Biomarkers Consortium, have convened for the same purpose of developing a consensus statement but have not yet published their findings.

The ESPEN defined sarcopenia as “a condition characterized by loss of muscle mass and muscle strength” (Muscaritoli et al. 2010). They introduce sarcopenia as a disease of the elderly but stipulate that its development may be associated with other conditions that are not exclusively seen in older persons like disuse (due to immobility, physical inactivity, bed rest…), malnutrition, neurodegenerative diseases and cachexia. Consequently, younger people can be sarcopenic especially those with inflammatory diseases.

The EWGSOP defined sarcopenia as “a syndrome characterized by progressive and generalized loss of skeletal muscle mass and strength with a risk of adverse outcomes such as physical disability, poor quality of life and death” (Cruz-Jentoft et al. 2010). To assess the severity of sarcopenia, muscle strength and physical performance are added to the muscle mass evaluation. These authors suggested a conceptual staging as ‘presarcopenia’, ‘sarcopenia’ and ‘severe sarcopenia’ (see table 1). The ‘presarcopenia’ stage is characterized

by low muscle mass without impact on muscle strength or physical performance. The 'sarcopenia' stage is characterized by low muscle mass, plus low muscle strength or low physical performance. 'Severe sarcopenia' is the stage identified when all three criteria of the definition are met (low muscle mass, low muscle strength and low physical performance).

Table 1. EWGSOP conceptual stages of sarcopenia (Cruz-Jentoft et al. 2010).

Stage	Muscle Mass		Muscle Strength		Physical Performance
Presarcopenia	-				
Sarcopenia	-		-	Or	-
Severe Sarcopenia	-	And	-	And	-

EWGSOP recognizes sarcopenia as a condition with many causes and varying outcomes and although sarcopenia is mainly observed in older people, it can also develop in younger adults. Moreover, this group suggests recognizing sarcopenia as a geriatric syndrome. Based on the identification of the cause of sarcopenia, two categories are proposed. Sarcopenia can be considered 'primary' (or age-related) when no other cause is evident but aging itself, while sarcopenia can be considered 'secondary' when one or more other causes are evident (see table 2). In many older people, the etiology of sarcopenia is multi-factorial so that it may not be possible to characterize each individual as having a primary or secondary condition.

Table 2. Suggested categorization of sarcopenia by EWGSOP (Cruz-Jentoft et al. 2010).

Primary Sarcopenia	
Age-related sarcopenia	No other cause evident except aging
Secondary Sarcopenia	
Inactivity-related sarcopenia	Can result from bed rest, sedentary lifestyle, deconditioning or zero gravity conditions
Disease-related sarcopenia	Associated with advanced organ failure (heart, lung, liver, kidney, brain), inflammatory disease, malignancy or endocrine disease
Nutrition-related sarcopenia	Results from inadequate dietary intake of energy and/or protein, as with malabsorption, gastrointestinal disorders or use of medications that cause anorexia

IWGS defines sarcopenia as “the age-associated loss of skeletal muscle mass and function” (Fielding et al. 2011). Sarcopenia is presented by these authors as a multifactorial syndrome that can include disuse, altered endocrine function, chronic disease, inflammation, insulin resistance, and nutritional deficiencies.

SSCWD provides a definition more directly applicable in the clinical world. Indeed, these authors decided that “sarcopenia with limited mobility” would be an acceptable term to define persons with a need for therapeutic intervention and presented it as a syndrome not a disease (Morley et al. 2011). Finally, sarcopenia with limited mobility was defined as “a person with muscle loss whose walking speed is equal to or less than 1 m/s or who walk less than 400 m during a 6 minutes walk test”. The limitation in mobility should not be clearly attributable to the direct effect of specific disease (e.g. peripheral vascular disease, dementia or cachexia).

1.5. Convergences and differences of the various definitions

Although all these definitions are different, they present a high level of agreement in some aspects of sarcopenia.

1.5.1. Sarcopenia as a syndrome not a disease

In the literature, sarcopenia can be presented as an age-related process of normative aging, a disease or a syndrome.

Among these four groups, only the ESPEN considers the sarcopenia as a disease of the elderly whereas the other three groups present it as a syndrome. It is thus clear that sarcopenia (or “sarcopenia with limited mobility”) is a syndrome but there is still a debate around the fact of considering it as only a geriatric syndrome.

Indeed, although the four groups agree that sarcopenia is strongly related to age, they also agree on the fact that other factors not related to age (e.g. malnutrition, bed rest, cachexia, and endocrine disease) could be the cause of sarcopenia in subjects not considered old. EWGSOP would speak about a secondary sarcopenia as described previously. On the other hand, a minority of SSCWD would support the use of the term ‘‘myopenia’’ to indicate the presence of clinically relevant muscle wasting owing to any illness at any age (Morley 2007; Fearon et al. 2011) and would reserve the use of ‘‘sarcopenia’’ for older persons. Some have argued that the term dynapenia is better suited to describe age-associated loss of muscle strength and function. Finally, sarcopenia is already a widely recognized term, so replacing it might lead to further confusion (Cruz-Jentoft et al. 2010).

The term of geriatric syndrome refers to a frequent, complex and expensive condition at the origin of the deterioration of the health during aging. The consideration of several criteria is generally used to consider a set of clinical signs characterizing a geriatric syndrome. These criteria include prevalence of these signs at the elderly, multifactorial causes as well as the negative consequences which these clinical signs have on the physical independence of the individual. Sarcopenia represents an impaired state of health with a high personal toll- mobility disorders, increased risk of falls and fractures, impaired ability to perform activities of daily living, disabilities, loss of independence and increased risk of death (Cawthon et al. 2007; Lauretani et al. 2003; Rolland et al. 2008; Topinková 2008; Hartman et al. 2007).

With regard to these various criteria, it thus seems obvious that sarcopenia must be considered as a real geriatric syndrome as supported by EWGSOP but some particularly situations may raise doubts this.

1.5.2. Not only muscle mass

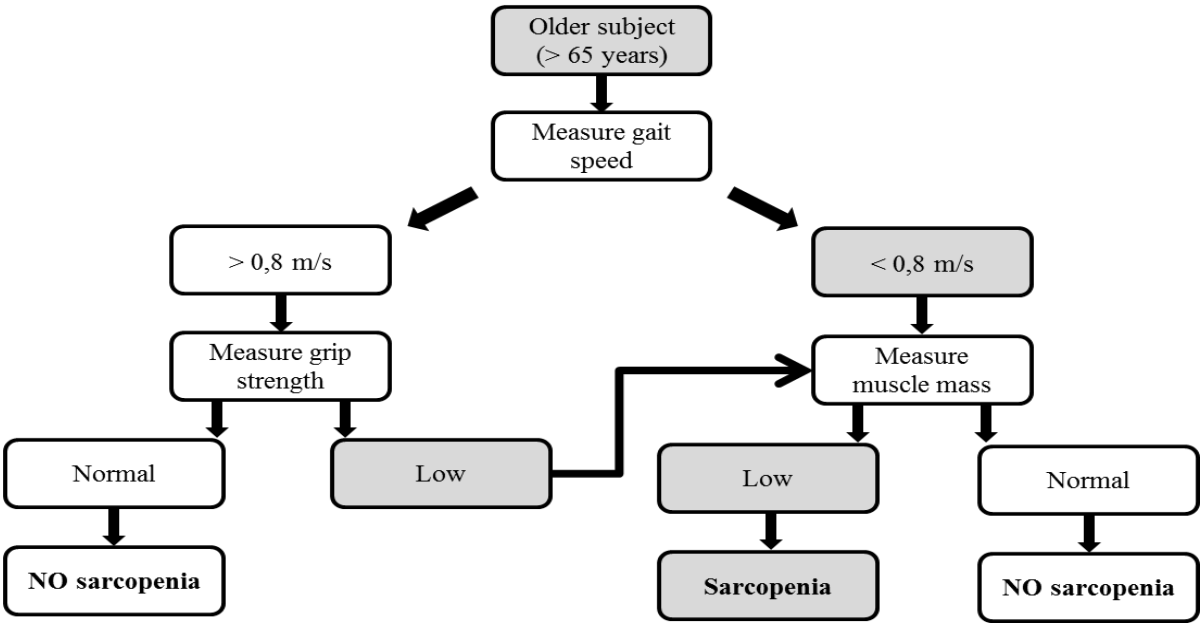
The clinical relevance of sarcopenia depends on its being a marker of impaired outcomes, mortality being the most striking, but perhaps not the most relevant. Physical disability is a major concern in old people (Cruz-Jentoft 2012), and from a practical point of view, appears as a more relevant outcome. Furthermore, as presented previously, numerous studies showed that the muscular mass is a weak predictor of outcome (Visser et al. 2000; Visser et al. 2005; Newman et al. 2006; Gale et al. 2007; Hairi et al. 2010; Goodpaster et al. 2006) and that the relation between muscle mass and muscle function (strength and power) is not linear (Goodpaster et al. 2006; Janssen 2004). Thus, measurement of muscle strength and/or physical performance appears essential parameters in the diagnosis of sarcopenia because they reflect the actual physical capacity of the individual to deal with demands of everyday life. This is why, the four groups all added besides the muscular mass at least a criterion of physical performance and/or muscular function. All groups suggest a criterion based on walking speed and only EWGSOP recommends also assess muscle strength but does not specify a method to use. Muscle fatigue could be another parameter in the diagnosis of sarcopenia but there is no standardized tool to evaluate it.

1.5.3. Diagnosis and strategy of case finding

Identifying subjects with sarcopenia, both for clinical practice and for selection of individuals for clinical trials, seems to be an important task.

ESPEN suggests diagnosing sarcopenia when two criteria are fulfilled: a low muscle mass and a low gait speed. For their part, normal muscle mass is defined using data derived from young subjects aged 18–39 years from the Third NHANES population (Janssen et al. 2002), and the requirement for a diagnosis of sarcopenia is the presence of a muscle mass ≥2 standard deviations below the mean of this reference population. This value can normally be calculated automatically by equipment such as DXA. A low gait speed is defined as a walking speed below 0.8 m/s in the 4-m walking test (Guralnik et al. 2000). However, this working group provides no guidance on the population that would need to be evaluated. As mentioned earlier, EWGSOP suggested diagnosing sarcopenia when at least two of three criteria apply: low muscle mass, low muscle strength, and/or low physical performance. To diagnose sarcopenia, these authors have developed a gradual approach based on gait speed measurement as the easiest and most reliable way to begin sarcopenia case finding or screening in practice (Figure 1) and chose a cut-off point of >0,8 m/s (identified as a predictive risk factor for adverse outcomes, Abellan van Kan et al. 2009). Here, all people aged over 65 should be evaluated starting with the measure of gait speed. If it is strictly lower than 0,8m/s, grip strength will be performed. In the case of a normal value, people are considered as non sarcopenic. On the other hand, muscle mass will be evaluated. If it reaches a low value, people are considered as sarcopenic. Otherwise, people are considered as non sarcopenic. Cut-off point for grip strength and muscle mass depend on the measurement technique chosen and this is probably why EWGSOP just provides a table with some of them extracted from articles.

Figure 1. EWGSOP-suggested algorithm for sarcopenia case finding in older individuals (Cruz-Jentoft et al. 2010).



For IWGS, diagnosis of sarcopenia should be based on having a low whole body or appendicular fat free mass in combination with poor physical functioning. Current methods index appendicular fat free mass to height squared or whole body fat free mass to height squared. In patients with poor functional capacity, most easily identified using gait speed of than 1 m/s, sarcopenia can be diagnosed when the lean mass is less than 20% tile of values for healthy young adults. Currently objective cut points can be made for sarcopenia in men at an appendicular fat free mass/ ht^2 of ≤ 7.23 kg/m^2 and in women at ≤ 5.67 kg/m^2 (Newman et al. 2003). For these authors, presence of sarcopenia should be evaluated in older patients (no age specified) who have clinically observed declines in physical functioning, strength, or health status. Sarcopenia should also be considered in patients who present difficulties in performing activities of daily living, have a history of recurrent falls, have documented recent weight loss, have recently been hospitalized, or have chronic conditions associated with muscle loss (e.g. Type II diabetes, chronic heart failure, chronic obstructive pulmonary disease, chronic kidney disease, rheumatoid arthritis, and malignancies). Sarcopenia should be considered in patients who are bedridden, non-ambulatory, or who cannot rise from a chair unassisted. In addition, for patients who are ambulatory and can arise from a chair, gait speed should be assessed across a 4 meter course. Patients with a measured gait speed less than 1.0 m/s should be referred for body composition assessment using whole body DXA.

SSCWD use the term "sarcopenia with limited mobility" and diagnose it when "a person with muscle loss whose walking speed is equal to or less than 1 m/s or who walk less than 400 m during a 6 minutes walk test". The person should also have a lean appendicular mass corrected for height squared of more than two standard deviation below that of persons between 20 to 30 years of age of the same ethnic group (Morley et al. 2011). This working group recommends that all patients older than 60 years who are falling, who feel that their walking speed has decreased, who were recently hospitalized, who have been on prolonged bed rest, who have problems arising from a chair, or who need to use an assistive device for walking should be screened for sarcopenia with mobility impairment.

Again, there is no real consensus because diagnosis and strategy of case finding are directly linked with the definition used but two different approaches appear. One is based on screening the general population (EWGSOP) whereas the others look for identifying some risk groups (SSCWD and IWGS). The age to investigate the presence of sarcopenia is still in debate (EWGSOP: ≥65years; IWGS: ≥60years) but would be around the sixties. For any given parameter included in a definition, there is a need to identify cutoff points that separate normal from abnormal values. The choice of cutoff values is arbitrary by nature, as it depends upon the measurement technique and the reference population chosen. There is not yet well-defined reference population but the trend would be to use a normative (healthy young adult) rather than other predictive reference population, with cutoff points at two standard deviations below the mean of healthy persons between 20 to 30 years of age of the same ethnic group. For the parameters directly related to the diagnosis of sarcopenia, all authors agree on assessing muscle mass and employing gait speed to assess physical performance. EWGSOP recommends completing physical performance assessment by measuring muscle strength.

1.6. Prevalence of Sarcopenia

Currently, the prevalence of sarcopenia varies extensively when different definitions, instruments of measurements, reference population (when one is used), skeletal muscle mass expression, methods of determining cutoff values are considered. This fact supports the need for a universal consensus of sarcopenia with full considerations of the aforementioned factors.

First, the prevalence of sarcopenia will depend on the used definition. Recently, Abellan van Kan et al. (2013) applied to the EPIDOS French cohort (3,025 women aged 75 years and older) six different definitions commonly used in literature (Baumgartner et al. 1998; Newman et al. 2003; Delmonico et al. 2007; Cruz-Jentoft et al. 2010; Fielding et al. 2011; Morley et al. 2011). Definitions based only on muscle mass (Baumgartner et al. 1998;

Newman et al. 2003; Delmonico et al. 2007) showed a higher prevalence than definition taking into account muscle mass, strength and physical performance (Cruz-Jentoft et al. 2010; Fielding et al. 2011; Morley et al. 2011). In the first case, values range from 9,4% to 18,8% whereas in the second case they are ranged between 3,3% and 14,2%. Very recently, authors have shown that the values obtained with the EWGSOP definition are higher than those obtained by the definition of IWGS. This result persisted whatever the index of muscle mass used (Lee et al. 2013).

The technique used to measure muscle mass also influence the prevalence of sarcopenia. In the New Mexico Elder Health Survey, sarcopenia defined as ASM/height² and measured by bioelectrical impedance affected 20% of men between 70 and 75 years, 50% of those over 80 years and between 25 and 40% in women in the same age groups (Baumgartner et al. 1998). Using DXA, the same authors published data from the same population of 8.8% in women and 13.5% in men aged 60-69 years and 16% in women and 29% in men over 80 years (Baumgartner 2000).

Using the same definition but with two different reference populations (National Health and Nutrition Examination Survey III and Cardiovascular Health Study), Janssen et al. showed different results. In the first case, the prevalence of sarcopenia was lower in men than women (7% *vs* 10%) while the opposite occurred in the second case (17% *vs* 11%).

The prevalence values will vary depending on the used method to express muscle mass: ASM divided by height² or by size and fat (residual method) (Baumgartner et al. 1998; Newman et al. 2003; Coin et al. 2013; Figueiredo et al. 2013; Dufour et al. 2013; Lee et al. 2013); total muscle mass divided weight and multiplied by 100 (Janssen et al. 2002; Janssen 2004; Janssen 2006). Thus, in men over 70 years, prevalence data reached 13.5% using ASM/height² and 19.8% with the residuals method (Figueiredo et al. 2013). In the same way, Dufour et al. (2013) reported prevalence values of 19% among men and 13% among women with ASM/height² and a value of 25% for men and women with residuals. From these studies, it appears that ASM/height² would be better to use with underweight people while residuals method would be more appropriate with normal and overweight people.

On the other hand, the used methods to determine cutoff values can influence the prevalence of sarcopenia. For example, one Italian group applied to the same population (men and women aged between 20 and 80 years) three different cutoff values for ASM/height² (Coin et al. 2013). The first cutoff points were obtained by subtracting 2 SDs from the mean ASM/height² value for their 20-39 years old healthy subjects. With these cutoff points (6.54 kg/m² in men and 4.82 kg/m² in women), prevalence of sarcopenia was 0% in men and 0,3%

in women. In the second case, the 15th percentile of the distribution of the ASM/height² for their young population (corresponding to about 1 SD below the mean) was used. Then, the cutoff points for sarcopenia were 7,59 kg/m² in men and 5.47 kg/m² in women, giving rise to a prevalence of 19.2% and 12.6%, respectively. In the third case, the cutoffs were obtained instead for an elderly population (older than 65 years) using the 20th percentile of the distribution of the ASM/height² (Health ABC Study: white and black American men and women aged 70 to 79 years, Delmonico et al. 2007). A cutoff of 7.64 kg/m² in men and 5.78 kg/m² in women was obtained. Then, prevalence of sarcopenia was 20% for both genders.

Finally, compared with the classical definition of sarcopenia, modern diagnostic criteria added considerations of muscle strength and physical performance to the muscle mass, which lowered the prevalence of sarcopenia (Abellan van Kan et al. 2013; Lee et al. 2013).

2. Making a Diagnosis of sarcopenia

To diagnose sarcopenia and the degree of it, it should be based on specific indicators of muscle mass and strength as well as physical performance. One of the current problems is to determine these parameters as precisely as possible. This part is devoted to outline the different measurement techniques in humans and rodents that can be implemented to diagnose sarcopenia. Table 4 resumes all these techniques.

2.1. Muscle mass assessment

Table 3 resumes the most used methods to assess muscle mass which are well reviewed in the following papers: Woodrow 2009; Lustgarten & Fielding 2011; Cooper et al. 2013.

Three imaging techniques can be used to estimate muscle mass or lean body mass of a person: computed tomography (CT) scan, magnetic resonance imaging (MRI) and DXA. CT and MRI are the most precise imaging systems and the only able to measure fat infiltration and non-contractile components into skeletal muscle and therefore determine muscle quality (Simoneau et al. 1995; Kent-Braun et al. 2000). Despite their cost, these methods are the actual gold standards for estimating muscle mass in research.

Then, DXA constitutes an attractive alternative method both for research and for clinical use to distinguish fat, bone mineral and lean tissues (Cruz-Jentoft et al. 2011) because it is cheaper, faster and expose to a lesser levels of radiation than MRI and CT with a good precision. Unfortunately, the equipment is not portable which may preclude its use in large

scale epidemiological studies (Chien et al. 2008).

Thanks to validated prediction equations for multiethnic and baseline populations, men and women, including the elderly (Roubenoff et al 1997. Janssen et al 2000) make bioelectric impedance (BIA) a good tool for epidemiological studies and clinical practice. The test is perfectly appropriate for both ambulatory and bedridden patients as many of the elderly are.

Anthropometric measures (e.g. skinfold thickness, calf circumference) can be possibly used to evaluate body composition but related-age changes of fatty deposits and loss of skin elasticity contribute to generate errors in older populations. Finally, anthropometric measures are considered as not relevant in the elderly because of the risk of confusion in the analysis of these parameters (Rolland et al. 2008).

In the context of research carried out in rodents, the mass of one or several muscles (soleus, gastrocnemius) or cross sectional areas (CSA) are conventionally measured post-mortem. Generally, these estimations are considered as reference methods for sarcopenia studies but imaging techniques or BIA usually used in humans are more and more used in rodents.

Table 3. Muscle mass assessment technics (adapted from Cruz-Jentoft et al. 2011).

Methods	Advantages	Drawbacks	Principal Field of application
TC and MRI	Gold Standard Muscle quality assessment	Very Expensive Qualified personal requirement High radiation exposure (CT) Few equiments No immediate results	Investigation
DXA	Moderate cost Moderate radiation exposure Very good precision No experimented personal	Not portable No information about muscle quality Influenced by hydration status No immediate results	Clinical practice Investigation
BIA	Inexpensive Good precision Portable (bedridden patients) No radiation exposure No experimented personal Immediate results	No information about muscle quality Less sensitive than earlier techniques Influenced by hydration status	Clinical practice Epidemiological studies
Anthropometry	Inexpensive Easy to realize Portable (bedridden patients)	Low precision and sensibility Difficulty in interpreting the results	Neither

CT: computed tomography; MRI**:** magnetic resonance imaging; BIA: bioelectric impedance

2.2. Strength assessment

The assessment of muscle strength (the maximum capacity of a muscle to generate force in a very short time) is now a parameter in its own right of diagnosis of sarcopenia. There are fewer well-validated techniques to measure muscle strength. On the other hand, the muscle fatigue (defined as ‘‘the inability of the muscle to generate or maintain the levels of strength required for a given work rate’’ by Vøllestad 1997) is also a parameter which should be taken into account in the diagnosis of this syndrome (Theou et al. 2008). Indeed, the activities of daily life ensuring independence of elderly or inactive person require maintaining or repeating submaximal muscular effort and rarely produce maximum muscle effort (Petrella et al. 2005). Again, cost, availability and ease of use can determine whether the techniques are better suited to clinical practice or are useful for research. It must be remembered that factors unrelated to muscle (e.g. motivation or cognition) may hamper the correct assessment of muscle strength.

In humans, lower limbs strength can be measured under isometric or isokinetic conditions. The assessment of maximal isometric strength is usually measured as the maximum force applied to the ankle (Edwards et al. 1977). Assessment of muscle fatigue can be performed by determining the force-holding time curve during isometric contraction for a given percentage of the maximum force (e.g. 40%). Nevertheless, choice of isokinetic conditions appears more relevant but did not appear functional because they required the subject to consistently achieve maximum effort until fatigue, which is not really a task performed by elderly people in their daily life (Lindström et al. 1997). This is why more recent studies assess muscle fatigue under isotonic conditions by measuring for example the ability to maintain or repeat an exercise as quickly as possible for a given sub maximal strength (McNeil & Rice 2007). Nowadays, isokinetic dynamometers (e.g. Cybex) permit to assess isometric, isotonic and isokinetic strength, as the couple concentric strength developed at different angulations (Hartmann et al. 2009). Some data are now available in older populations for maximum strength and muscle fatigue in isotonic or isokinetic condition (Neder et al. 1999; Goodpaster et al. 2001). If isokinetic appears appropriate for research, its use in clinical practice is limited due to a specific and expensive equipment requirement.

Although lower limbs are more relevant than upper limbs for gait and physical function, handgrip strength has been widely used and is well correlated with most relevant outcomes. Isometric hand grip strength is strongly related with lower extremity muscle power, knee extension torque and calf CSA (Lauretani et al. 2003). Thus, low handgrip strength is a

clinical marker of poor mobility and a better predictor of clinical outcomes than low muscle mass (Lauretani et al. 2003). In practice, there is also a linear relationship between baseline handgrip strength and incident disability for activities of daily living (Al Snih et al. 2004). Finally, low cost, availability and ease of use make this method is widely used in both clinical practice and research.

In rodents, grip strength tests are a widely-used non-invasive method designed to evaluate mouse limb strength. It is based on the natural tendency of the mouse to grasp a bar or grid when it is suspended by the tail. During these tests the mouse grips with both forelimbs and/or hind-limbs a single bar or a mesh. Three different tests are commonly used. The Mesh Grip Test measures the ability of the mouse to remain clinging to an inverted or tilted surface such as a wire grid or a cage lid for a period of time. This test shows that the muscle endurance is altered at 24 months in rats (Joseph et al. 1983; Spangler et al. 1994). The Wire Grip Test (or Rod suspension test) measures the ability of the mouse to hang on a wire with its forepaws for a preset length of time or until grip fails. This test appears to be a useful indicator for the diagnosis of sarcopenia since the time of suspension in rats from 22-24 months decreases (Spangler et al. 1994; Goettl et al. 2001). Finally, with the Automatic Grip Strength (AGS) the mouse grasps a horizontal metal bar or grid while is pulled by the tail. The bar or grid is attached to a force transducer that peak pull-force achieved on its digital display. The AGS is the unique noninvasive test giving a numeric value. In the three tests, the value obtained has to be relativized by the animal weight.

However, the strength and muscle fatigue are generally assessed invasively. More specifically, the muscle is removed and the tendon ends are connected to a dynamometer and two electrodes. A suitable electric current is sent in order to generate a maximum tetanic stimulation considered developable maximum force by the muscle. Muscle fatigue is itself estimated by the difference in maximum force developed by the muscle between the beginning and 4-5 minutes from electrical stimulation (Ryall et al. 2007; Ljubicic & Hood 2009). These approaches in animals have the advantage of assessing the intrinsic muscle strength, regardless of neural factors.

2.3. Physical performance assessment

The gait speed is now the recommended parameter to assess physical performance to diagnose sarcopenia (Cruz-Jentoft et al. 2010; Fielding et al. 2011; Morley et al. 2011) but others test provided specifically for elderly people are also accepted. The most commonly used are the Short Physical Performance Battery (SPPB) (standardized battery of short physical tests), the timed Get-up-and-go (TUG) or the Stair climb power test (SCPT).

Gait speed is usually evaluated by the six meters test recommended by IWGS and SSCWD (Fielding et al. 2011; Morley et al. 2011) or the four meters test recommended by EWGSOP (Cruz-Jentoft et al. 2010). Cutoff points for sarcopenia are defined as a speed lesser than 1m/s in first case and lesser than 0,8m/s in the second case (Cesari et al. 2009). Gait speed can be used in clinical practice and research. The SPPB evaluates balance, gait speed, strength and endurance by examining an individual's ability to stand with the feet together in side-by-side, semi-tandem and tandem positions, time to walk 8 feet and time to rise from a chair and return to the seated position five times (Guralnik et al. 1994). Each event allows get a performance score and the sum of the scores of all tests provides an overall performance. A score below 8 is in favor of sarcopenia (Guralnik et al. 2000). SPPB is a standard measure for research and clinical practice. The TUG is a test to measure the time required to perform a series of basic motor tasks. The subject must stand up from a chair, walk a short distance, turn around and come back to sit. It allows the estimation of the dynamic balance that is assessed on a scale of 1 to 5 (Mathias et al. 1986). A score below 3 would be in favor of sarcopenia (Mathias et al. 1986). Finally, the SCPT used clinically estimates the power of the lower limbs (Bean et al. 2007). The subject must perform the rise of 10 markets as soon as possible. The power of the lower limbs is then calculated in relation to the height of the market, the rate of rise and standardized with the weight of the subject (Bean et al. 2007). It may be useful in some research settings but cutoff point in sarcopenia context needs to be not defined.

In rodents, a number of tests are also available to assess the physical performance in old animals (Table 4). One of them consists in measuring the time that the rodent can stay in balance on a narrow beam (Beam Balance Test) or a tightrope (tightrope test). A significant reduction in maintenance time is observed in rats from 23-24 months testifying alterations in the balance and coordination of the animal (Altun et al. 2007; Emerich et al. 2008). As previously described, Mesh Grip, Wire Grip and Auto Grip strength tests can be used to evaluate muscle function. Endurance capacity can be assessed by maximal aerobic speed tests (Derbré et al. 2012) or maximal oxygen consumption tests (Høydal et al. 2007).

Table 4. Summary of methodologies used to assess muscle mass, muscle strength and physical performance in humans and rodents.

Measured parameter	Humans	Rodents
Muscle Mass	- Computed Tomography (CT) - Magnetic resonance imaging (MRI) - Dual energy X-ray absorptiometry (DXA) - Bioelectrical impedance (BIA)	- Weighing muscle after sacrifice - Cross sectional area post mortem - Technics used in humans
Muscle Strength	- Handgrip strength - Knee flexion/extension (e.g. Cybex)	- Auto Grip Strength Meter (noninvasive) - Electrostimulation (very invasive)
Physical Performance	- Short Physical Performance Battery (SPPB) - Gait Speed - "Timed get-up-and-go" test (TUG) - Stair climb power test (SCPT)	- Wire Grip test and Mesh Grip tests - Beam balance test, tightrope test and rotarod test - Maximal aerobic speed test - Maximal oxygen consumption

3. Muscle characteristic changes during aging leading to sarcopenia

3.1. Loss of muscle mass

It is considered that a reduction of about 40% of the CSA of occurs between 20 and 80 years in humans (Doherty et al. 1993; Vandervoort 2002). Works on the topic are mainly based on data obtained from the lower limbs using various techniques mentioned above (see table 3). *Via* ultrasound imaging, Young et al. (1985) reported such reductions of 25 to 35% beyond the quadriceps CSA in elderly men by an average of 30-70 years. Similar results were observed by CT in the quadriceps (Klitgaard et al. 1990) and in the biceps and triceps (Rice et al. 1989; Klitgaard et al. 1990). These results are also confirmed by measurements made directly on the CSA post-mortem muscle with a decrease of approximately 40% in elderly subjects on average 20 to 80 years (Lexell et al. 1988).

Rodents, especially rats, are experimental animal models particularly useful for the study of sarcopenia. Depending on the species, rats are considered as aged between 18 and 30 months (Hopp 1993). Fischer 344 Brown Norway F1 hybrid rats with a higher life expectancy than other species of rats (40 months) are one of the most used specie to study sarcopenia. In this strain, a decrease from 30 to 50% by weight of the gastrocnemius was observed between 6 and 30 months (Haddad et al. 2006; Hofer et al. 2008; Marzetti, Wohlgemuth, et al. 2008; Siu et al. 2008). The Wistar strain has also been very well used. A significant reduction in muscle mass is observed after 24 months in mixed fiber type muscles such as gastrocnemius (Capel et al. 2004; Mosoni et al. 2004). Significant decreases were also reported in this species in the soleus muscle from 28 months old animals (Mosoni et al. 2004; Degens et al.

2008). Usually, nevertheless the strain, around 18 months (middle age for rats), the weight of the soleus, extensor digitorum longus (EDL), gastrocnemius but also quadriceps, tibialis anterior and plantaris is reduced compared to animals aged 6 or 12 months (Kimball et al. 2004; Paturi et al. 2010; Ibebunjo et al. 2013). This decrease is relatively slow and low at 18 months (about 10%) but accelerates thereafter to reach -30 to -40% at 24 months (old age) (Kimball et al. 2004; Paturi et al. 2010; Ibebunjo et al. 2013). In very old animals, this decrease can reach up to 60% in some muscles notably the gastrocnemius (Kimball et al. 2004; Ibebunjo et al. 2013).

Skeletal muscles are heterogeneous at the level constituent muscle fibers. Physiological properties, such as contractile speed, resistance to fatigue, metabolism, mitochondria myoglobin content and ATPase activity and various enzyme content vary among types of muscle fibers (see table 6). In skeletal muscle, it is possible to distinguish four major fiber types, called type I, IIa, IIx and IIb, based on the presence of specific myosin heavy chain (MyHC) isoforms: MyHC-I, MyHC-IIa, MyHC-IIx and MyHC-IIb (Schiaffino & Reggiani 2011). These fibers also differ in oxidative/glycolytic metabolism. These four fiber populations are present in mice, rats and many other mammalian species, however only type I, IIa and IIx fibers are present in human muscles (Smerdu et al. 1994). In addition, intermediate hybrid fibers, containing type I and IIa, or IIa and IIx, or IIx and IIb MyHCs, are frequent in normal muscles (DeNardi et al. 1993) and become more numerous whenever fiber type shifts take place (Klitgaard et al. 1990; Maier et al. 1988; Patterson et al. 2006).

The age-related decrease in muscle mass is mainly due to a loss of muscle fibers affecting both fiber types I and II (Young et al. 1985; Aniansson et al. 1986; Lexell et al. 1988). While a decrease of only 5% of the number of fibers occurs between 24 and 50 years, a reduction of 30 to 40% is reported between 50 and 80 years (Aniansson 1992). These results in reduction of about 1% per year of the total CSA beyond 50 years (Kent-Braun 1999; Frontera et al. 2000b). However, atrophy of the muscle fiber (reduction of its diameter) is also implicated in the decrease of muscle mass associated with age (Aniansson et al. 1986; Lexell et al. 1988; Lexell and Downham 1992). Atrophy does not affect similarly all types of muscle fibers. Indeed, it is the fast type II fibers that appear to be most affected by aging, with a decline from 20 to 60% of their size (Larsson et al. 1978; Essen-Gustavsson and Borges 1986; Lexell et al. 1988; Singh et al. 1999; Hikida et al. 2000). This phenomenon seems differentiated itself in different type II fibers with larger reductions in fiber IIb and IIx type compared to type IIa fibers (Aniansson et al. 1986; Coggan et al. 1992).

The possible mechanisms underlying the atrophy of muscle fibers of older people will be developed later.

Table 5. Muscle fibers specificity and impact of aging on their atrophy.

Muscle fibers specificity	Type I	Type II Type IIa	Type IIx	Type IIb
Color	Red	Red	White	White
MHC isoform	MyHC-I	MyHC-IIa	MyHC-IIx	MyHC-IIb
Contractile speed	Slow	Fast	Fast	Fast
Fatigue resistant	High	High	Low	Low
Dominant Metabolism	Oxidative	Oxidative	Glycolitic	Glycolitic
Mitochondria and myoglobin content	High	High	Low	Low
ATPase Activity	Low	Low	High	High
Age-related atrophy	+	++	+++	+++

3.2. Loss of muscle strength

The decrease in muscle strength is a key criterion to identify sarcopenia (Cruz-Jentoft et al. 2010). Muscle strength of the knee extensors is important to consider due to its functional importance (Doherty 2003). On average, the peak strength is reduced by 20 and 40% between 20 and 70-80 years (Larsson 1979; Murray et al. 1985; Young et al. 1985). Similar results are observed for other muscle groups such as shoulder and wrist flexors (McDonagh et al. 1984; Bassey and Harries 1993). Larger reductions (50%) are still reported in subjects aged over 90 years (Murray et al. 1980; Murray et al. 1985). Decreased muscle strength seems to be accelerated especially between 60-70 years. Indeed, longitudinal studies observed a reduction of 30 to 40% of the peak strength of the knee and shoulder extensors between 60 and 70 years (Aniansson et al. 1986; Frontera et al. 2000a; Hughes et al. 2001).

Although muscle fatigue is not part of the parameters used in the diagnosis of sarcopenia, it is important to focus on its evolution during aging because the more a person will be easily tired the least it will be independent in carrying out daily activities. Aging also can affect muscular fatigue. However data from different muscle groups from young and elderly people do not permit to pronounce a real consensus. Indeed, some studies have found that older people exhibited less fatigue than their younger counterparts during isometric or isokinetic contractions (Hakkinen 1995; Hunter et al. 2005; Yassierli and Nussbaum 2007) while others observed no difference (McNeil and Rice 2001; Lanza et al. 2004; Theou et al. 2008a). These results could be explained by the fact that the absolute maximum forces developed by the elderly in this type of exercise are lower than those developed by young people (Yassierli and Nussbaum 2007), and also by the selective fiber type II atrophy

observed during the aging (as described above). Studies in isotonic conditions are fewer but report an increase around 10% of the muscle fatigue during aging (Hunter et al. 2005; McNeil and Rice 2007). The differences between all these works can be explained both by the exercise protocols used (type and duration of contraction, muscle groups) and populations evaluated (age, sex, level of physical activity). Further work appears necessary to clarify the effects of age on muscle fatigue.

In rodents, muscle strength is commonly assessed by invasive technics (as previously described). With aging, there is a decrease in the maximal force but the onset of this phenomenon seems to be different following the strain and the age of the rodent. Thus, in Fisher 344 Brown Norway F1 hybrid, a decrease in maximal force is generally observed between 32 and 36 months in soleus, gastrocnemius and extensor digitorum longus (EDL) (Brooks 1988; Ryall et al. 2007; Thomas et al. 2010). In Wistar rats, no difference appears in the EDL but maximal force decreased after 24 months in the soleus. Just like humans, studies focusing on the effects of aging on muscle fatigue reported conflicting results. Thus, Ljubicic & Hood (2009) observed a higher decrease of the maximal force of the tibialis anterior during aging after a fatigue protocol whereas others do not find any difference with aging (Ryall et al. 2007).

4. Chapter 1 abstract

In 1931, MacDonald Critchley was the first to recognize that muscle mass decreases with aging (Critchley 1931) and fifty-seven years later, Irwin Rosenberg called this phenomenon sarcopenia (Rosenberg 1989). The components of its definition have been in debate in the medical and scientific world. However, the different working groups agree on some points which can constitute the current consensus as follow. Sarcopenia is a geriatric syndrome initially characterized by a decrease in muscle mass that will get worse causing a deterioration in strength and physical performance (Muscaritoli et al. 2010; Cruz-Jentoft et al. 2010; Fielding et al. 2011; Morley et al. 2011). Some important questions are still under debate. What people should be primarily target for a diagnosis? What would the standardized diagnostic?

Thus, in humans as in rodents, aging is accompanied by a decrease in muscle mass around 40% from the adulthood to the death (Lexell et al. 1988; Kimball et al. 2004; Ibebunjo et al. 2013). The age-related decrease in muscle mass is mainly due to a loss of muscle fibers affecting both fiber types I and II (Young et al. 1985; Aniansson et al. 1986; Lexell et al. 1988) and an atrophy which affects particularly fast type II fibers (Larsson et al. 1978; Essen-Gustavsson and Borges 1986; Lexell et al. 1988; Singh et al. 1999; Hikida et al. 2000). In humans and rodents, parallel to the muscle mass decrease, it is noted a decrease in muscle strength during aging (Murray et al. 1985; Frontera et al. 2000; Brooks 1988; Ryall et al. 2007) which can reach more than 50%. Although still under debate, it seems that aging is also accompanied by an increase in muscle fatigue (Hunter et al. 2005; McNeil and Rice 2007).

As it will exposed in the next part, maintaining muscle mass and strength is under control numerous mechanisms that will be altered with aging leading to sarcopenia.

Chapter 2: Sarcopenia-related cellular and molecular skeletal muscle alterations

The development of effective treatments or strategies to prevent and/or fight against sarcopenia requires understanding the cellular and systemic mechanisms, and the underlying pathways involved in its onset and development. Maintaining muscle mass is first a balance between protein synthesis and protein degradation systems. Equilibrium between apoptosis and regeneration processes is also involved in maintaining muscle mass. The vital functions carried out by mitochondria in the context of energy provision, redox homeostasis, and regulation of several catabolic pathways confer these organelles a central role in the maintenance of myocyte viability.

In the following chapter, we will first describe the different pathways involved in protein turnover and some aspects of mitochondrial function and homeostasis when muscle mass is at equilibrium (no gain and no loss). In a second time, we will describe the alterations of these functions involved in the onset and development of sarcopenia.

1. Cellular and molecular mechanisms controlling proteins synthesis and degradation

An equilibrated balance between protein synthesis and protein degradation systems is necessary to maintain muscle mass. Protein synthesis and degradation are regulated by different pathways presenting some cross-talks.

1.1. Protein synthesis

Protein synthesis is the result of the transfer of biological information between the three biological polymers: DNA (deoxyribonucleic acid), RNA (ribonucleic acid) and proteins (Crik 1970; Crikc 1958). The three transfers common to all cells are replication (DNA synthesizes DNA), transcription (DNA synthesizes RNA) and translation (RNA synthesizes protein). Thus, protein synthesis will depend on the transcriptional and translational activity.

1.1.1. Transcriptional activity of muscle fiber

Transcriptional activity of muscle cell depends on the transcriptional capacity determined by the amount of DNA available and on the efficiency of transcription of target genes in each myonucleus.

From a theoretical point of view, DNA amount necessary to sustain gene transcription depends on the number of myonuclei which constitutes the major determinant of transcriptional capacity, and therefore a key issue to the success of protein synthesis. Myonuclei of mature myofibers are considered to be post-mitotic. In this context, supplemental DNA can be only brought by satellite cells. These cells located between the basal lamina and the sarcolemmal membrane are normally in a quiescent state (Mauro 1961), and can be activated to proliferate and then fuse with a pre-existing fiber or possibly reconstruct a new fiber (Hawke and Garry 2001; Charge and Rudnicki 2004). Having a high DNA content confers a high gene transcription capacity, but the activation of genes encoding muscle specific proteins and their transcription into mRNA (messenger RNA) are dependent on numerous transcription factors.

Among these latter, myogenic regulatory factors (MRFs), including MyoD, myogenin, Myf5, and MRF4, have been originally described to play major role in myogenesis (Olson et al. 1991) but seem to be also involved in the activation of genes encoding muscle proteins. Indeed, *in vitro* these transcription factors are able to transform fibroblasts into myoblasts (Rhodes and Konieczny 1989). *In vivo*, these MRFs promote muscle mass, and therefore the construction of contractile material (Bamman et al. 2007; Hyatt et al. 2008). The calcineurin/NFAT (Nuclear Factor of Activated T cells) signaling pathway also regulates the transcriptional activity by promoting a slow genetic program and consequently would promote the transcription of genes encoding certain muscle proteins such as myosin heavy chain type 1 (Delling et al. 2000).

1.1.2. Translational activity of muscle fiber

The translation of mRNA leading to protein synthesis is determined by two factors, translational efficiency and capacity. The translational efficiency could be defined as the protein synthesis per unit of total RNA, whereas the translational capacity is mainly determined by the ribosome content per unit of tissue (Millward et al. 1973). The PI3K/Akt/mTOR signaling pathway (see figure 2) is the main pathway regulating protein synthesis in skeletal muscle and is involved in the regulation of both sides of the translation of mRNA into protein (Nader et al. 2005).

1.1.2.1. An overview of the PI3K/Akt/mTOR signaling pathway

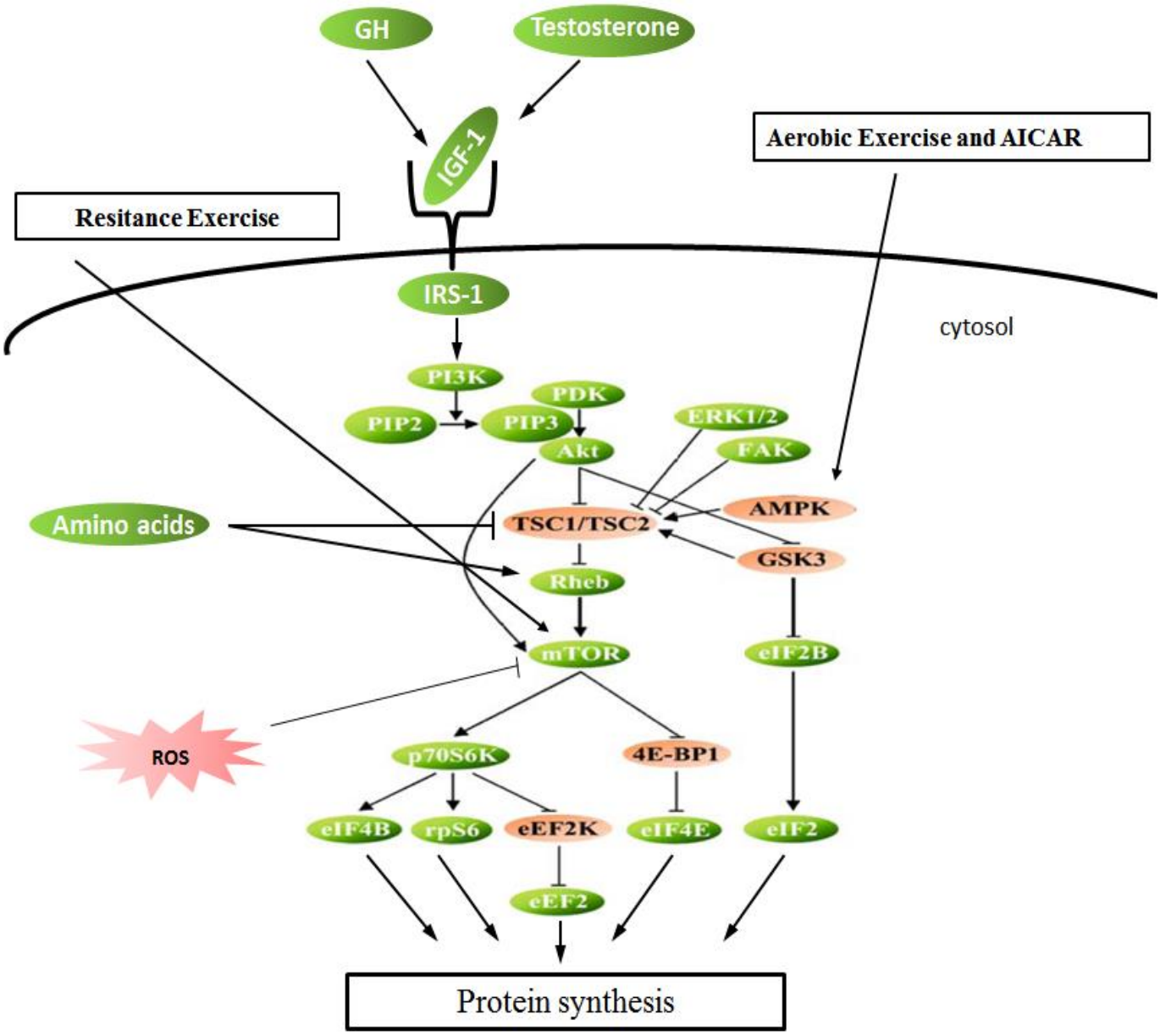
IGF-1 (Insulin-like Growth Factor I) is a secreted growth factor similar to insulin and may induce muscle hypertrophy through its anabolic effects. The IGF-1 induces an increase in protein synthesis by binding to its receptor IGF1R. This connection allows the phosphorylation of the receptor, which is necessary for the recruitment of its substrate IRS1 (insulin receptor substrate 1) (sun et al 1991). Its leads to stimulate PI3K protein (phosphatidylinositol-3-kinase) which catalyzes the transfer of a phosphate group on PIP2 (phosphoinositide-(4,5)-biphosphate) for generating PIP3 (phosphoinositide-(3,4,5)-triphosphate) (Schiaffino and Mammucari 2011) which in turn activate PDK-1 (phosphoinositide-dependent kinase-1), which finally will activate Akt (protein kinase B) (Schiaffino and Mammucari 2011; Andjelković et al. 1997; Vivanco and Sawyers 2002).

Then, Akt inactivates the TSC1 (Tuberous Sceloris protein 1)/TSC2 (Tuberous Sceloris protein 2) complex (Inoki et al 2003) allowing the Rheb GTPase (Ras homolog enriched in brain) to stimulate the mTOR protein (Huang and Manning 2009) which regulates protein synthesis (Schiaffino and Mammucari 2011). Finally, insulin and IGF-1 can also stimulate mTOR by the MAP kinase ERK1/2 (Extracellular signal Regulated Kinase 1/2) (Miyazaki et al. 2011) and Focal Adhesion Kinase (Durieux et al. 2007).

Once activated, mTOR will stimulate ribosome biogenesis, initiation and elongation of translation by activating the 70-kDa ribosomal protein S6 kinase (p70S6K) and by inhibiting 4E-BP1 (eukaryotic initiation factor 4E binding protein 1) (for a complete review see Shah et al. 2000; Wullschleger et al. 2006; Yang et al. 2008). mTOR controls the translation initiation by regulating the level of phosphorylation of 4E-BP1 protein (eIF-4E binding protein 1), a repressor of eIF4E and by phosphorylating p70S6K which in turn leads to activation of eIF4B. Moreover, once activated, p70S6K also inhibits the eEF2K factor which in turn cancel the repressive effect of this latter on eEF2 resulting in the elongation of translation. On the other hand, p70S6K is involved in the ribosome biogenesis through the activation of the ribosomal protein rpS6 (ribosomal protein S6) which stimulates ribosome protein synthesis. Ribosome biogenesis is also directly controlled by mTOR which promotes transcription of ribosomal genes (for review see Martin & Hall 2005; Wullschleger et al. 2006). All these steps will lead to protein synthesis.

Protein synthesis can also be directly promoted by Akt through the GSK-3 factor (Glycogen Synthase Kinase 3) inhibition by phosphorylation, which promotes through activation of eIF2B (eukaryotic Initiation Factor 2B) factor (Welsh et al. 1998).

Figure 2. Overview of the PI3K/Akt/mTOR (inspired by Favier et al. 2008).

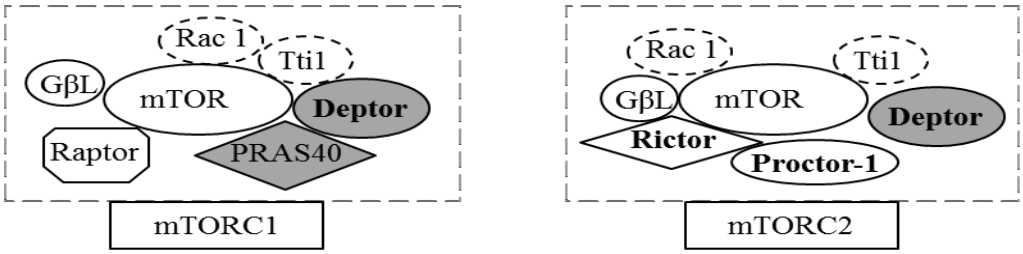


1.1.2.2. PI3K/Akt/mTOR pathway regulation

As previously described, PI3/Akt/mTOR pathway is regulated by growth factors such as IGF-1 and insulin and naturally by their up-streams such as testosteron and growth hormone leading to protein synthesis (Hayashi & Proud 2007; Kovacheva et al. 2010). However, other signals are also able to control this pathway by targeting in particular mTOR (see figure 2).

mTOR is a conserved serine threonine kinase that nucleates 2 distinct complexes mTORC1 and mTORC2 as shown in figure 3 (Laplante and Sabatini 2009). While mTORC1 is sensitive to the immunosuppressant drug rapamycin and is involved in protein synthesis, mTORC2 in general is not (Dowling et al. 2010; Oh et al. 2010; Tato et al. 2011). For this reason, we will focus only on mTORC1 which we call by default mTOR.

Figure 3. mTORC1 and mTORC2 complexes representation (modified from Adegoke 2012).



Aerobic exercise or chemical exercise mimetic such as AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside) are able to decrease mTOR activation through the cellular energetic sensor AMPK (AMP-activated protein Kinase) (Hardie 2003; Thomson et al. 2008; Leick et al. 2008; Leick, Lyngby, et al. 2010; Leick, Fentz, et al. 2010). A decrease in the AMP/ATP ratio like occurring during aerobic exercise leads to AMPK phosphorylation and restores energy homeostasis by activating catabolic processes and repressing anabolic processes such as protein synthesis by mTOR inhibition (Hardie 2008; Shaw 2009). AMPK acts through TSC2 (Inoki et al. 2003), or directly on mTOR (Cheng et al. 2004; Gwinn et al. 2008)leading to p70S6K and 4EBP1 phosphorylation inhibition (Bolster et al. 2002; Williamson et al. 2006; Thomson et al. 2008).

On the other hand, conversely to aerobic exercise, resistance (strength) exercise activates the PI3K/Akt/mTOR pathway (Tannerstedt et al. 2009; Witard et al. 2009; Camera et al. 2010; Adegoke 2012). Resistance exercise increases muscle protein synthesis in parallel with elevated AKT phosphorylation (Dreyer et al. 2008). Functional overload-induced hypertrophy in rodent muscles occurs in parallel with increased phosphorylation of mTOR (Reynolds et al. 2002) and of AKT (Spangenburg et al. 2008). Moreover electrical stimulation induces phosphorylation of the mTOR substrate p70S6K1 (O'Neil et al. 2009). Nevertheless, the involvement of AMPK in exercise-induced muscle anabolism, and that such an effect is at least in part related to mTOR, can be inferred from recent studies that show that myotubes deficient in AMPK (Lantier et al. 2010) or muscle from mice lacking AMPK (Mounier et al. 2009) are bigger in size.

Few data also suggest that reactive oxygene species (ROS) such as H_2O_2 appear to impair mTOR assembly and therefore preventing mTOR-mediated phosphorylation of 4E-BP1 (Zhang et al. 2009). Moreover, oxidative DNA damage are known to activate p53 which is able to inhibit mTOR *via* AMPK and TSC2 (Feng et al. 2005). These effects could be triggered by the stress-responsive molecules REDD1 (Regulated in Development and DNA damage responses 1) and REDD2 (RTP801/DDIT4 and RTP801L/DDIT4L, respectively) which decrease the activity of mTOR by activating TSC2 (Brugarolas et al. 2004; Corradetti et al. 2005; Favier et al. 2010; Murakami et al. 2011).

mTOR activation is also controlled by the availability of amino acids (AAS) (Kimball & Jefferson 2010). Indeed, amino acid starvation, in particular leucine leads to a decrease of p70S6K and 4EBP1 (Hay and Sonenberg 2004). Moreover, administration of branched chain amino acids such as leucine (Hara et al. 1998) is sufficient to activate the mTOR pathway and enhance protein synthesis. Amino acids would activate mTOR through TCS1/TCS2 inhibition or by stimulating Rheb protein (Gao et al. 2002; Smith et al. 2005; Long et al 2005).

A new form of PGC-1α (PGC-1α4), which results from alternative promoter usage and splicing of the primary transcript, has been recently identified as involved in muscle growth, as shown by the finding that mice with skeletal muscle specific transgenic expression of PGC-1α4 show increased muscle mass and strength (Ruas et al. 2012). In cultured muscle cells, PGC-1α4 was found to induce IGF-1 and repress myostatin, thus promoting myotube hypertrophy, which was blocked by an IGF1 receptor inhibitor (Ruas et al. 2012). Thus, it would not be surprising if PGC-1α4 activates Akt/mTOR pathway but it remains to be demonstrated.

1.2. Proteolysis systems

Various systems autophagy are involved in protein degradation such as autophagy, Ca^{2+}-dependent pathways (i.e. calpains and caspase) and the ubiquitin-proteasome system (UPS). Activation of the cell's proteolytic systems is transcriptionally regulated, and a subset of genes that are commonly up- or down-regulated have been identified in atrophying skeletal muscle. These common genes are thought to regulate the loss of muscle components, and were thus designated atrophy-related genes or 'atrogenes' (Sacheck et al., 2007). Among the up-regulated atrophy-related genes are transcripts belonging to the Ca^{2+}-dependent pathways, UPS and autophagy systems that are currently accepted as the two systems most involved in skeletal muscle proteolysis (Sandri 2010; Powers et al. 2012; Bonaldo and Sandri 2013; Schiaffino et al. 2013; Sandri 2013).

1.2.1. Ca^{2+}-dependent pathway: calpains and caspases

The calpain system is a protein-degradation pathway of eukarotic cells composed of two enzymes: calpains and their endogenous inhibitor calpastatin which regulates their activity (Dargelos et al. 2008). Such proteases are calcium-dependent and non-lysosomal cysteine proteases (Dargelos et al. 2008). Originally, calpains were first presented as cleaving only the proteins that anchor the actin-myosin complex (e.g. titin, nebulin...) (Koh & Tidball 2000; Purintrapiban et al. 2003). However, it has been demonstrated that calpains specifically cleave the MHC-IIb isoform (Samengo et al. 2012). In the same way, Smuder et al. (2010) showed that exposure of myofibrils to hydrogen peroxide increases susceptibility of MHC and actin to be cleaved by calpains. Consequently, the susceptibility of myofibrillar proteins to calpain-mediated cleavage appears to be influenced by their prior oxidative modification. Calpains activity is regulated by several factors, including cytosolic calcium levels and the concentration of their inhibitor calpastatin (Goll et al. 2003).

Caspases are cytoplasmic cysteine-proteases that can cleave other proteins. Caspase-3 seems to be able to degrade the actin-myosin complex. Indeed, Du et al. (2004) have shown that purified and activated caspase-3 can cleave actin, breaking the muscle actin-myosin complex.

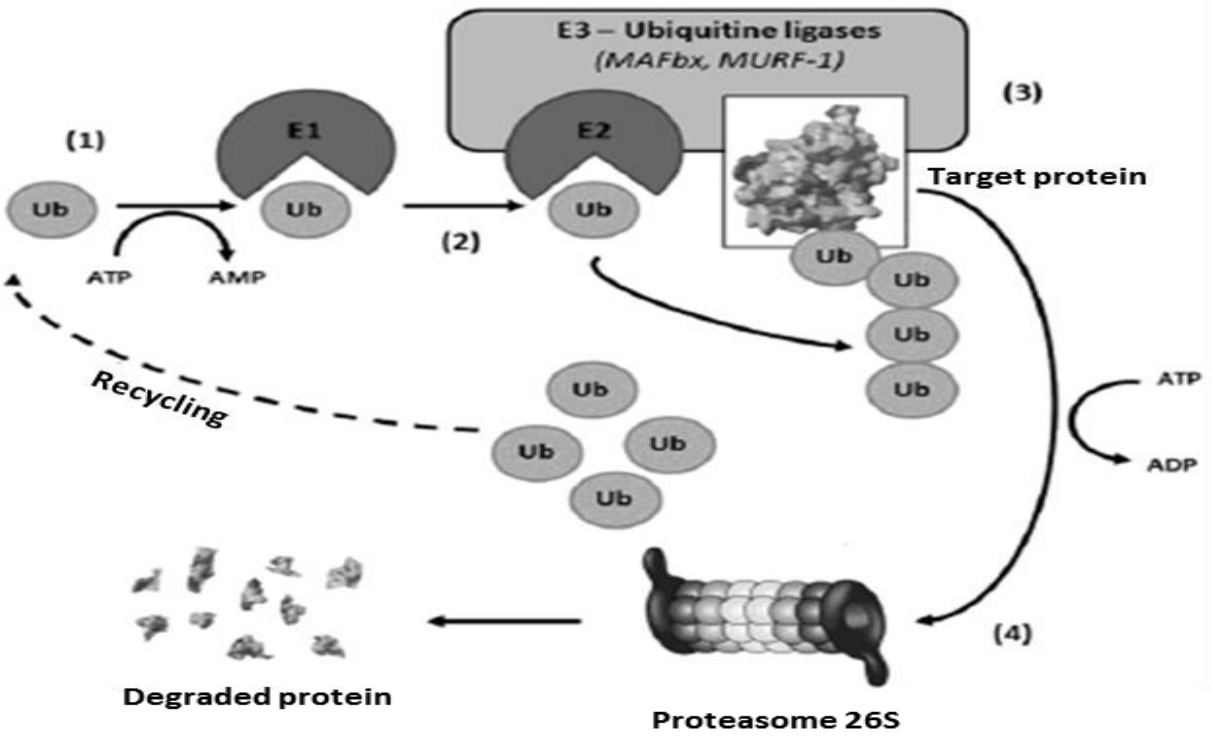
Calpains and caspases cannot degrade proteins into amino acids or smaller peptides but cleaved-protein by these latter will be degraded by UPS and autophagy.

1.2.2. Overview of the ubiquitine-proteasome-dependent system

In skeletal muscle, the UPS is required to remove sarcomeric proteins upon changes in muscle activity. This system is an organized process parted in two successive stages (see figure 4). Proteins must be fixed to a polyubiquitin chain (polyubiquitination stage) before being recognized and degraded by the 26S proteasome.

Polyubiquitination involves three enzymes (see figure 4): (1) E1 enzymes (activating enzyme) activate ubiquitin (Ub) proteins after the cleavage of ATP. (2) The ubiquitin is then moved from E1 to members of the E2 enzyme class (conjugating enzyme). (3) The ubiquitin is finally fixed to the target protein (e.g. myosin) by an E3 enzyme (ubiquitin ligase, e.g. MuRF1 and MAFbx) leading to the formation of a polyubiquitinated chain. This is the rate-limiting step of polyubiquitination, which affects the subsequent proteasome-dependent degradation. (4) Once the protein is ubiquitinated, it is docked to the 26S proteasome for degradation.

Figure 4. Ubiquitin-proteasome system.



Among the known E3s, only a few of them are muscles specific and up-regulated during muscle loss (Sacheck et al. 2007). Two E3s specifically expressed in striated and smooth muscles are MAFbx (also known as atrogin-1) and MuRF1 (muscle-specific RING-finger protein 1) (Bdolah et al. 2007; Bodine et al. 2001; Gomes et al. 2001). MuRF1 ubiquitinates several muscle structural proteins, including troponin I (Kedar et al. 2004), myosin heavy chains (Clarke et al. 2007; Fielitz et al. 2007), actin (Polge et al. 2011), myosin binding protein C and myosin light chains 1 and 2 (Cohen et al. 2009). MAFbx promotes degradation of MyoD, a key muscle transcription factor, and of eIF3-f, an important activator of protein synthesis (Csibi et al. 2010; Tintignac et al. 2005). Moreover, MAFbx would be involved in sarcomeric proteins degradation, including myosins, desmin, and vimentin (Lokireddy et al. 2012).

Ultimately, MAFbx would be a proteolytic actor capable of suppressing the process of protein synthesis, while MuRF1 would lead to proteolysis of myofibrillar proteins (Attaix & Baracos 2010). Presumably, several other E3s are activated during amyotrophy that promote the clearance of soluble cell proteins and limit anabolic processes and are presented in the following table (for a complete review see Schiaffino et al. 2013).

Table 6. Ubiquitin ligases and their role in skeletal muscle and muscle cell other than MuRF1 and MAFbx.

Ubiquitin ligase	Role in muscle and muscle cell
Trim 32	Thin filament degradation (actin, tropomyosin and troponins), α-actinin and desmin (Cohen et al. 2012)
CHIP	Filamin C (A Z-line protein) degradation (Arndt et al. 2010)
TRAF6	Involved in the optimal activation of various molecules such as AMPK (ref 97)
MUL1	Mitochondrial network remolding (ref 101 102)
FBxo40	Involved in IRS-1 degration (Shi et al. 2011)
Nedd4	Involved in unloading and denervation hypertrophy-induced (Koncarevic et al. 2007)

Trim 32: tripartite motif-containing protein 32; CHIP: Carboxy terminus of Hsc70 interacting protein; TRAF6: Tumor necrosis receptor-associated factor; MUL1: mitochondrial E3 ubiquitin protein ligase 1; FBx40: F-box only protein; Nedd4: Neural precursor cell expressed developmentally down-regulated protein 4.

1.2.3. Overview of Autophagy

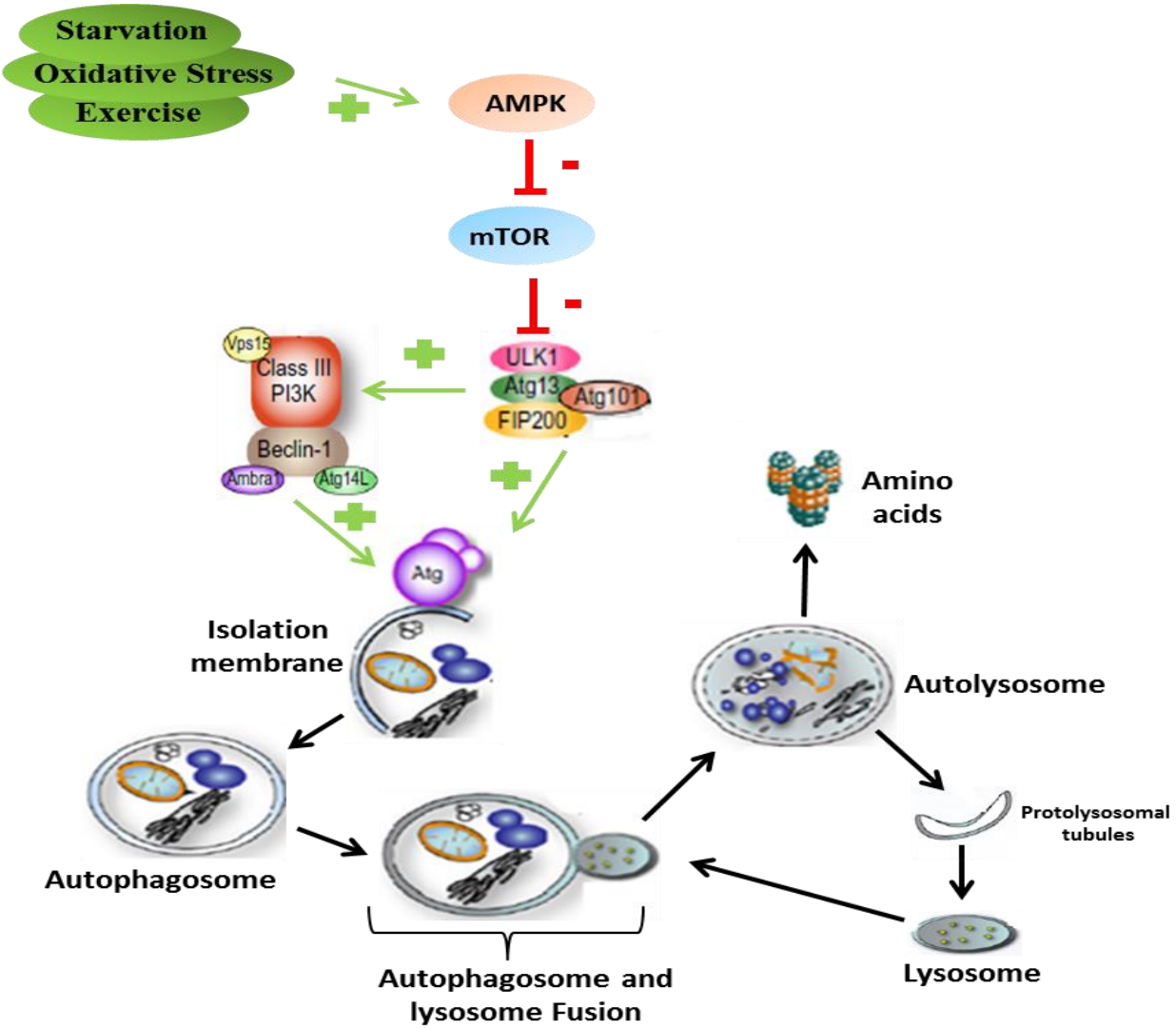
Autophagy is primarily a mechanism for cell survival. There are three types of autophagy: macro-autophagy, chaperone-mediated autophagy and micro-autophagy. It is still unknown whether micro-autophagy occurs in skeletal muscle. Most data on the role of the autophagic process in muscle are related to macro-autophagy. Macro-autophagy (hereafter referred to as autophagy) is a highly regulated lysosomal pathway for the degradation of non-myofibril cytosolic proteins, macromolecules and organelles (Zhao et al. 2007, Mizushima 2007; Sandri 2010).Autophagy whose mechanisms are resumed in the figure 5 (for review see Levine & Klionsky 2004; Rautou et al. 2010) is highly regulated by the autophagic gene (Atg) protein family (see table 7; for review see Mizushima 2007). Autophagy can be activated by numerous signals such as starvation, caloric restriction, hypoxia, oxidative stress, exercise, DNA and mitochondria damage (Liu et al. 2008; Kroemer et al. 2010; Mazure & Pouysségur 2010; Wohlgemuth et al. 2010; Kim et al. 2013).

Table 7. Equivalent Atg proteins between yeast and mammals and their functions (extracted from Mizushima 2007).

Name in Yeast	Name in Mammal	Function
Atg 1	ULK1, 2	Atg1/Atg13/Atg 17/Atg 29 complex : Autophagy initiation
Atg 2	Atg 2	Atg 2/Atg18/Atg9 complex : Autophagosome Formation
Atg 3	Atg 3	E2-like enzyme specific for Atg 8: Autophagy induction
Atg 4	Atg 4, 4B, Autophagin 3,4	Cystein protease: Cleave the C-terminal part of Atg 8
Atg 5	Agt 5	Atg 12/Atg 5/Atg 16 complex: Autophagosome formation
Atg 6	Beclin-1	Sub-unit of the complex Vsp34 PI3K: Autophagosome formation
Atg 7	Atg 7	E1-like Enzyme specific for Atg 8 and Atg 12
Atg 8	LC3, GABARAP, GATE-16	Autophagosome Formation
Atg 9	Atg 9L1,L2	Atg 2/Atg 19/Atg 9: autophagosome formation
Atg 10	Atg 10	E2-like enzyme specific for Atg 12: Autophagy induction
Atg 11		Only in yeast
Atg 12	Atg 12	Atg 12/Atg 5/Atg 16 complex
Atg 13	Atg 13	Autophagy induction

Autophagic signals lead to AMPK-induced mTOR inhibition leading to Ulk1 (Unc-51-like kinase 1) complex activation (Eskelinen & Saftig 2009) allowing the Beclin-1/Class III PI3K complex activation (Cao & Klionsky 2007; Sandri 2013). These phenomena stimulate Atg protein such as LC3-1 and LC3-2 leading to autophagic vesicles formation called autophagosomes. Then, fusion of autophagosomes and lysosomes leads to the formation of autolysosomes. This steps seems to control by a lysosomal membrane protein Lamp-2 (Huynh et al. 2007). This fusion leads to the exposure of autophagosome contents (i.e., cytosolic proteins) to lysosomal proteases (e.g. cathepsins) resulting in proteolytic degradation (Bechet et al. 2005).

Figure 5. Autophagy proteins degradation mechanisms (inspired by Rautou et al. 2010).



A specific mitochondria autophagy (called mitophagy) occurs to degrade these latters when they are damaged. In mammals, parkin, PINK1, Bnip3 and Bnip3L have been shown to regulate mitophagy. Inactivation of their genes leads to abnormal mitochondria (Bothe et al. 2000; Hara et al. 2006). PINK1 recruits parkin to mitochondria, where parkin promotes mitophagy through ubiquitination of outer mitochondrial membrane proteins that are recognized by p62, which brings autophagic vesicles to ubiquitinated mitochondrial proteins (Youle & Narendra 2011; Narendra & Youle 2011). Bnip3 and Bnip3L reportedly bind directly to LC3, and can therefore recruit the growing autophagosome to mitochondria (Hanna et al. 2012; Novak et al. 2010).

Emerging evidence suggests that a baseline level of autophagy is required for maintenance of normal muscle function and mass. Indeed, studies reveal that increases in autophagy above baseline contribute to skeletal muscle atrophy due to fasting, denervation or in the model of mechanical ventilation-induced diaphragmatic proteolysis (Mammucari et al. 2007; O'Leary & Hood 2009; Hussain et al. 2010). Mice with muscle specific inactivation of Atg clearly demonstrate the essential role of autophagy in muscle homeostasis. For example, muscle-specific knockout of Atg7 mice presents a dramatic skeletal muscle atrophy and weakness due to a decreased autophagosome formation (Masiero et al. 2009). Moreover, oxidative stress (OS) induced by the muscle-specific expression of a mutant superoxide dismutase protein (SOD1G93A) in mice causes muscle atrophy mainly by activating autophagy (Dobrowolny et al. 2008). Attenuation of autophagy by inhibition of LC3 preserves muscle mass in these transgenic mice (Dobrowolny et al. 2008). Furthermore, in atrophying muscle, the mitochondrial network is dramatically remodeled following fasting or denervation, and mitophagy *via* Bnip3 (Romanello et al. 2010; Romanello & Sandri 2013).

1.2.4. UPS and autophagy regulation

Forkhead box O (FoxO) transcription factors family members are known to up-regulate UPS and autophagy. Their activity is modulated (positively or negatively) by direct or indirect actions of co-factors and by interaction with other transcription factors. Several others pathways can up-regulate UPS and autophagy independently of FOXO.

1.2.4.1. Proteolysis systems FoxO dependent-regulation

The FoxO family members include three isoforms: FoxO1, FoxO3 and FoxO4. FoxOs activity is regulated by several post-translational modifications, including phosphorylation, acetylation and mono- and polyubiquitination (Huang & Tindall 2007). For example, when

FoxOs are phosphorylated, these transcription factors migrate from the nucleus to the cytosol where they lose their biological action. Conversely, when they are hypophosphorylated, FoxOs migrate from the cytosol to the nucleus where they are active (Calnan & Brunet 2008).

In rodent and human muscle, it has been shown that FoxO3 is responsible of the up-regulation of several Atg in skeletal muscle such as Bnip3, LC3 and PI3KIII (Zhao et al. 2007; Piétri-Rouxel et al. 2010; Hussain et al. 2010). On the other hand, FoxO members are able to up-regulate MAFbx and MuRF1 leading to muscle atrophy (Sandri et al. 2004).

Activation or repression of FoxOs are controlled by numerous factors. Here, we will only describe the action of Akt, PGC-1α, AMPK and nNOS which have a known role in sarcopenia. Positive and Negative known FoxOs family regulators are resumed in the table 8.

Akt, a very potent autophagy inhibitor in skeletal muscles, can phosphorylate all FoxOs promoting their export from the nucleus to the cytoplasm (Calnan & Brunet 2008). Acute activation of Akt in mice or in muscle cell cultures completely inhibits FoxO3 leading to autophagy inhibition during fasting (Mammucari et al. 2007; Mammucari et al. 2008; Zhao et al. 2007; Zhao et al. 2008). Moreover, Akt can block the up-regulation of MAFbx and MuRF1 in atrophying muscles (Stitt et al. 2004; Lee 2004; Sandri et al. 2004).

It has been shown in muscle cell and mice skeletal muscle that activation of AMPK induced by exhaustive exercise or AICAR treatment can stimulate FoxO3 which in turn will increases MAFbx and/or MuRF1 expression, and autophagy-related proteins such as LC3B-II and Beclin1 (Nakashima & Yakabe 2007; Romanello et. 2010; Sanchez et al. 2012; Pagano et al. 2014) leading to protein breakdown (Nakashima & Yakabe 2007).

PGC1-α and its homolog PGC1-β are able to inhibit the transcriptional activity of FoxO3 which leads to decrease protein breakdown and limits muscle atrophy during denervation, fasting, heart failure, aging by inhibiting autophagy and UPS degradation (Geng et al. 2011; Sandri et al. 2006; Wenz et al. 2009; Brault et al. 2010). For instance, Sandri et al. (2006) and Brault et al. (2010) showed that following denervation, transgenic mice overexpressing PGC-1α or PGC-1β specifically in muscle showed lower muscle atrophy due to a smaller increase in the expression of MAFbx and MuRF1 and a diminished autophagy. On the other hand, Wenz et al. (2009) showed that these same mice also presented a lesser active autophagy compared to wild type (WT) mice.

It has been shown that nNOS through NO production is able to enhance FoxO3-mediated transcription of atrogin- 1 and MuRF1 (Suzuki et al. 2007), and LC3 and Bnip3 (autophagy regulators) (Piétri-Rouxel et al. 2010). nNOS inhibition by two different inhibitors

(7-nitroindazol and N-nitro-l-arginine methylester) limited muscle loss during hindlimb and denervation (Suzuki et al. 2007).

Table 8. Positive and Negative known FoxOs family regulators.

Positive regulators of FoxOs family	Negative regulators of FoxOs family
AMPK (Greer et al. 2009)	Akt (Calnan & Brunet 2008)
REDD1 (Shimizu et al. 2011)	PGC-1α (Sandri et al. 2006)
nNOS (Piétri-Rouxel et al. 2010)	SGK1 (Andres-Mateos et al. 2013)
	JunB (Raffaello et al. 2010)
	Runx1 (Wildey & Howe 2009)

1.2.4.2. Proteolysis systems FOXO independent-regulation

Although FoxOs play a major role in the regulation of proteolytic systems, there are also independent FoxOs signaling pathways regulating proteolysis system through in particular the Tumor Necrosis Factor α (TNF-α).

The TNFα is known to activate the Nuclear Factor Kappa B (NFκB) pathways (Peterson et al. 2011). The activation of this transcription factor is sufficient to induce muscle atrophy, a phenomenon that could be explained in part by the specific overexpression MuRF1, but not MAFbx (Cai et al. 2004). Furthermore, TNF-like weak inducer of apoptosis (TWEAK), a member of the TNF superfamily, has been recently identified as involved in muscle atrophy through an activation of NFkB leading to increase MuRF1 expression (Dogra et al. 2007; Mittal et al. 2010). The lack of disruption of the expression of MAFbx by NFkB suggests that another signaling pathway may be involved in its regulation.

Indeed, in cultured myoblast and *in vivo*, Li et al. (2005) revealed that TNFα induces reactive oxygen species (ROS) production (in particular hydrogen peroxide; H_2O_2) which leads to mitogen-activated protein kinases (MAPK) p38 activation (phosphorylation). Activation of MAPK p38 then leads to increase MAFbx mRNA independently of NFkB. MAFbx up-regulation by p38 MAPK independently of Akt/FoxO and NFkB signaling pathways has been confirmed (Yamamoto et al. 2008). More recently, Mclung et al (2010) demonstrated that cachectic stimuli result in increased phosphorylation of p38 MAPK in cultured myotubes and in mice leading to activate UPS and autophagy-mediated muscle proteolysis and atrophy. Inhibition of p38 MAPK activity attenuates myotube atrophy *in vitro* with attenuated ubiquitin ligase and Atg expression (McClung et al. 2010).

Others molecules such as the PIP3 Jumpy (Romero-Suarez et al. 2010; Hnia et al. 2012), the pro-inflammatory Interleukine-6 (Llovera et al. 1997) and STAT3 (Signal Transducer and Activator of Transcription 3) (Bonetto et al. 2012) are also described to be involved in the proteolysis systems FoxO independent-regulation.

1.3. Myostatin: master regulator of muscle mass

Myostatin (Mstn) or GDF-8 (Growth Differentiation Factor-8), a member of the TGF-β superfamily (Transforming Growth Factor beta) is a major negative regulator of muscle growth that is expressed predominantly in skeletal muscle (Lee 2004).

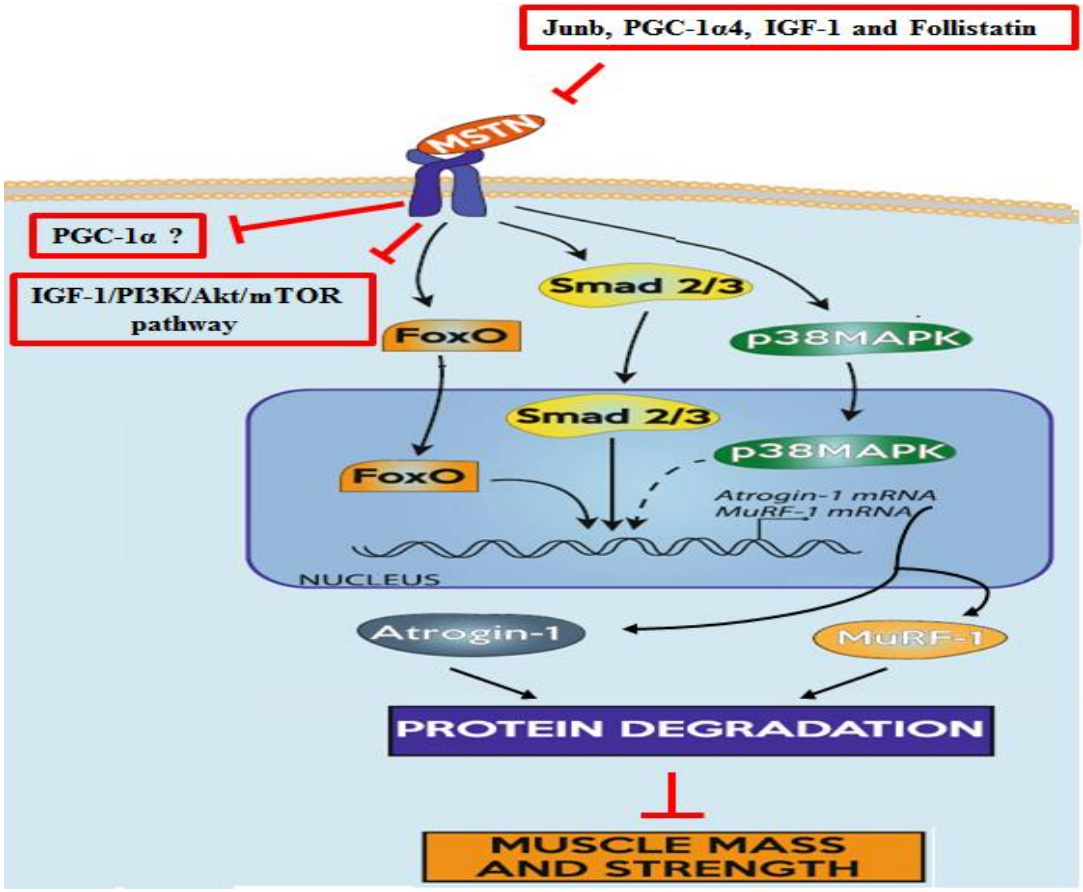
Mutations of the Mstn gene results in a hypertrophic phenotype as observed in cattle (Belgian Blue and Piedmontese breeds; McPherron & Lee 1997), in mice (compact hypermuscular mice breed, Szabó et al. 1998) and in human (a boy who presented a loss of function mutation in the human myostatin gene; Schuelke et al. 2004). This phenotype is due to both an increase in muscle fiber number (hyperplasia) and size (hypertrophy) at least in KO $Mstn^{-/-}$ mice (Amthor et al. 2009; Girgenrath et al. 2005; McPherron et al. 1997; Mendias et al. 2006; McPherron et al. 2009) and appears to be dose dependent: Heterozygous mutant mice have a milder increase in muscle mass than homozygous mutant mice. In addition to increased muscle mass, $Mstn^{-/-}$ mice have increased insulin sensitivity (Guo et al. 2009; Wilkes et al. 2009), reduced adipose tissue mass (Lin et al. 2002), and resistance to weight gain when fed a high-fat diet (McPherron & Lee 2002; Hamrick et al. 2006). On the other hand, $Mstn^{-/-}$ mice present altered contractile properties compared to $Mstn^{+/+}$ or $Mstn^{+/-}$ as shown by a decreased specific force and power production of muscle fibers (Mendias et al. 2011), a greater force deficit following two lengthening contractions (Mendias et al. 2006) and a higher muscle fatigue (Ploquin et al. 2012; Giannesini et al. 2013). Moreover, $Mstn^{-/-}$ mice tendons's appear to be smaller, more brittle, and more hypocellular than those of WT mice (Mendias et al. 2008). $Mstn^{-/-}$ mice also present lower maximal exercise capacity (Savage & McPherron 2010).

Myostatin is held in an inactive form in the muscle extracellular matrix, and when activated, it binds to its receptor (Kollias & McDermott 2008). It has been shown both *in vitro* and *in vivo* that Smad 2 and Smad 3 are the transcription factors mediating Msnt effects on muscle mass (Lokireddy et al. 2012; Sartori et al. 2009; Trendelenburg et al. 2009). Their downstream still remain to discover and also the Smad-dependent atrophy mechanisms. Until now, it has been demonstrated that Smads can regulate specific target genes but only in association with other DNA-binding cofactors (Massagué et al 2005) as the FoxOs family

(Gomis et al 2006). In addition, myostatin-Smad2/3 signaling can inhibit the IGF-1/PI3K/Akt/mTOR axis and reduce p70S6K activation (Amirouche et al. 2009; Sartori et al. 2009; Trendelenburg et al. 2009).On the other hand, the overexpression of myostatin may also decrease the expression of PGC-1α in skeletal muscle (Durieux et al. 2007). Myostatin have shown to be inhibited by Junb, PGC-1α4, IGF-1 and follistatin (Raffaello et al. 2010; Ruas et al. 2012; Gumucio & Mendias 2013). These different pathways are resumed in figure 6.

As described above, it is clearly demonstrated that myostatin inhibition leads to muscle hypertrophy however, the mechanism of myostatin activation and its role and capacity to trigger muscle atrophy remain unclear *in vivo*. However, intra-muscular Mstn local administration leads to marked muscle atrophy and a decreased force production in mice (Mendias et al. 2012). In the same way, Mstn treatment *in vivo* and *in vitro* induces cachexia (McFarlane et al. 2006). Transgenic mice overexpressing Mstn selectively in skeletal muscle have lower muscle mass (Reisz-Porszasz et al. 2003). Moreover, purified myostatin inhibits protein synthesis and reduces myotube size when added to differentiated myotubes in culture (Taylor et al. 2001).

Figure 6. Myostatin mechanism leading to muscle atrophy (inspired by Gumucio & Mendias 2013).



2. Role of Mitochondria in Cellular Homeostasis

The mitochondrion is an organelle lying in any eukaryotic cell, particularly in the muscle fiber. Its physiological role is crucial as it contributes to both regulation of calcium homeostasis, and cell cycle, force production, but primarily represents the main source of ATP in the cell (Calvani et al. 2013).

2.1. Mitochondrial biogenesis

The mitochondria consist of proteins encoded from both mitochondrial (mtDNA) and nuclear DNA (nDNA). Although mtDNA contains just 37 genes that encode 13 proteins (all within the electron transport chain; ETC), 2 ribosomal and 22 translational RNA, proper organelle biogenesis and function require input from both genomes. Several transcription factors and molecular regulators have been highlighted in orchestrating mitochondrial biogenesis (making of new mitochondrial proteins, Johnson et al. 2013) and substrate metabolism.

2.1.1. Mitochondrial biogenesis pathway

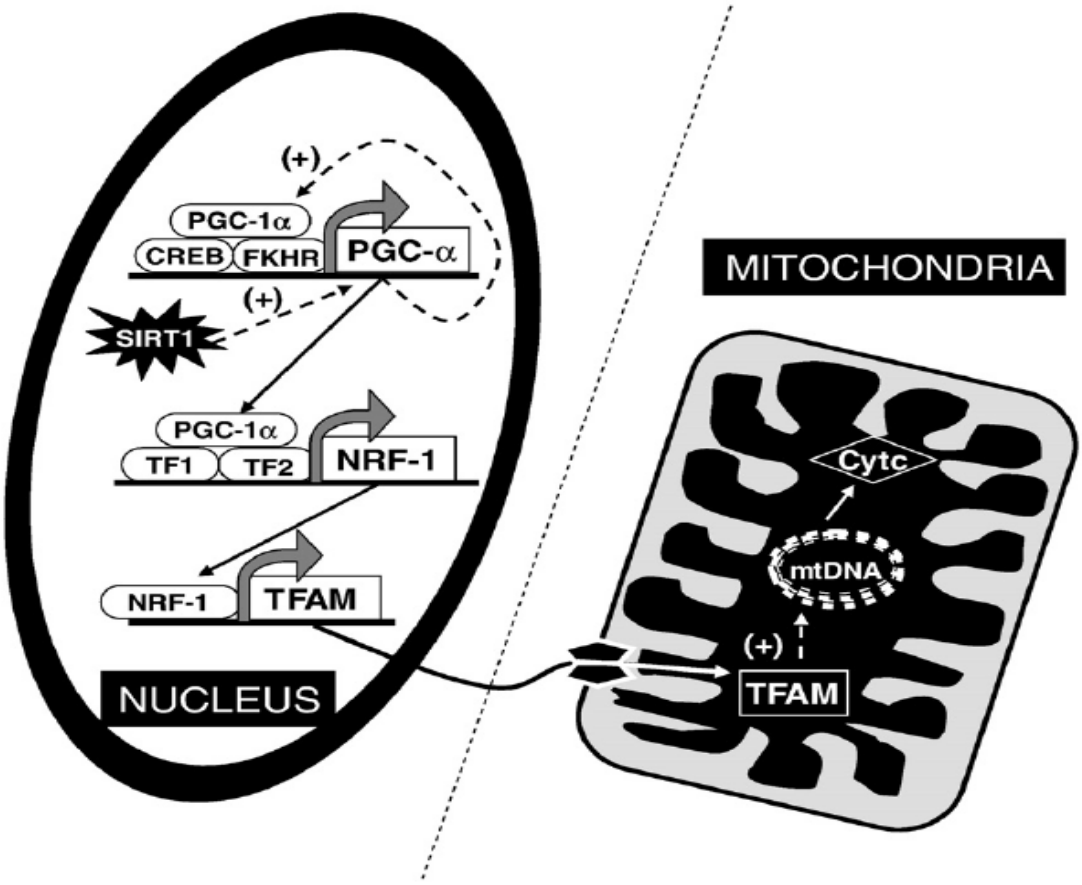
PGC-1α is considered as the master regulator of mitochondrial biogenesis from the set of transcription factors involved in this process (Puigserver et al. 2003; Viña et al. 2009). Works on knockout mouse models of PGC-1α (PGC-1α KO) or transgenic overexpressing PGC-1α specifically in muscle (MCK PGC-1α) have established the key role of this co-activator in mitochondrial biogenesis. Indeed, deletion of PGC-1α in muscle is clearly associated with a reduction in mitochondrial content and activity of key enzymes in mitochondrial function as citrate synthase (CS), succinate dehydrogenase (SDH) or cytochrome c oxidase I (COX I) (Adhihetty et al. 2009; Leick, Lyngby, et al. 2010; Leick, Fentz, et al. 2010). In contrast, chronic overexpression of PGC-1α in muscle leads to an increase of the same mitochondrial markers (Wenz et al. 2009; Brault et al. 2010).

PGC-1α does not directly regulate the expression of nuclear genes encoding mitochondrial proteins, but acts on others transcription factors (see figure 7) which serve as intermediaries in this regulation (Puigserver et al. 1999).

In the muscle cell, different molecules, stimulated mainly during muscle contraction, will activate the process of mitochondrial biogenesis (for review see Viña et al. 2009). These molecules will increase the activity of transcriptional factor PGC-1α leading to stimulate its own expression and the expression of the Nuclear respiratory factor 1 and 2 (NRF-1 and 2) genes (Hood et al. 2006). The latter will then stimulate the expression of nuclear genes

encoding mitochondrial protein. NRF-1 and NRF-2 increases the expression of TFAM (mitochondrial transcription factor A), which through various complexes of the mitochondrial protein import system (HSPs, TOMs, TIMs), will be carried into the mitochondrial matrix and stimulate the expression of 13 genes encoded by the mitochondrial DNA (Virbasius & Scarpulla 1994). The proteins encoded by the nuclear and mitochondrial genomes will then be assembled via specific proteins to form the various complexes of the electron transport chain necessary for the synthesis of ATP.

Figure 7. Schematic representation of the regulation of mitochondriogenesis (extracted from Viña et al. 2009).



Surprisingly, PGC-1α appears to not be mandatory for mitochondrial biogenesis in particular in response to aerobic training, known to induce mitochondrial biogenesis in rodent and human (Gomez-Cabrera, Domenech, Romagnoli, et al. 2008; Viña et al. 2009; Derbré et al. 2012). In fact, muscle-specific PGC1α knockout animals showed increased mitochondrial protein content following aerobic training in young mice (Leick et al. 2008). However, PGC-1α is required for training-induced prevention of age-associated decline in mitochondrial enzymes as citrate synthase in mouse skeletal muscle (Leick, Lyngby, et al. 2010).

2.1.2. Mitochondrial biogenesis pathway up-streams

Exercise, but also cold exposure (Derbré et al. 2012), chemical treatment such as AICAR (Winder et al. 2000), some natural compounds such as caffeine (Ojuka et al. 2003) and resveratrol (Lagouge et al. 2006), some pharmacological agents such as clenbuterol (Miura et al. 2007) or certain hormones such as thyroid hormone (Koulmann et al. 2008) are recognized as modulators of the mitochondrial biogenesis. As a result of this diversity, it was highlighted various regulatory molecules of PGC-1α and the most relevant are described below.

AMPK is involved in regulating the expression of PGC-1α. Indeed, injection of AICAR activate AMPK leading to an increase in mRNA of PGC-1 α in rodents (Jørgensen et al. 2005; Narkar et al. 2008; Leick, Lyngby, et al. 2010). AMPK also phosphorylates PGC-1α also which contributes to increase its activity (Jäger et al. 2007). Using KO of isoforms α1 and α2 AMPK mice, Jorgensen et al. (2005) have specifically shown that after exercise AMPK α1 is required to increase PGC-1α expression. These data are very surprising because protein synthesis requires ATP and is decreased with AMPK activation. Thus, AMPK may inhibit global protein synthesis while simultaneously increasing mitochondrial protein synthesis (Johnson et al. 2013).

Muscle contraction results in the activation of the family of mitogen-activated protein kinases (MAPK) (Aronson et al. 1997; Widegren et al. 1998). p38 MAPK appears involved in the regulation of PGC-1α since activation of p38 MAPK led to the phosphorylation and increased expression of PGC-1 α in various body tissues including skeletal muscle (Zhao et al. 1999; Puigserver et al. 2001; Cao et al. 2004). Moreover, Akimoto et al. (2005) demonstrated that the transcriptional control of PGC-1α by p38 MAPK required the phosphorylation of ATF2 (Activating transcription factor 2).

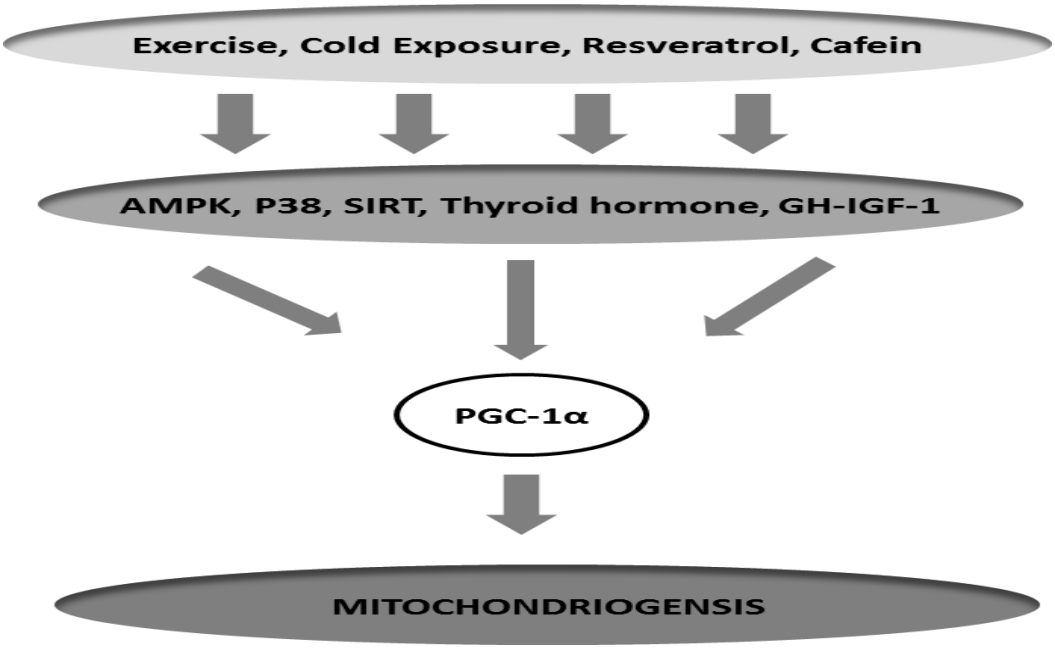
It was also observed in rodents that thyroid hormone treatment increased the expression of PGC-1α in skeletal muscle (Irrcher et al. 2003; Bahi et al. 2005; Koulmann et al. 2008; Derbré et al. 2012) *via* the activation of AMPK and p38 MAPK (Irrcher et al. 2003; Bahi et al. 2005; Kukuljan et al. 2009; Miklosz et al. 2012). On the other hand, Vescovo et al. (2005) reported in rats with right heart failure-related muscle atrophy that treatment with GH restores protein content of PGC-1α and cytochrome c involving IGF-1 and calcineurin (Vescovo et al. 2005). Similar data have been reported in the liver of aged rats treated with GH (Kireev et al. 2007). Later, Short et al. (2008) reported in human muscle that an infusion of GH for 14 h leads to an increase in the mRNA levels of TFAM and cytochome c and the activity of citrate synthase without increase in PGC-1α. In contrast, other authors reported no

improvement in mitochondrial respiration in young rats treated with GH (Peyreigne et al. 2002). In addition, growth hormone (GH) receptor knockout (GHRKO) (mice known to be remarkably long-lived) mice report an increase in numerous markers involved in mitochondrial biogenesis in the kidney but not in skeletal muscle (Gesing et al. 2011). In view of all these data, it appears that further studies are needed to confirm or not the involvement of growth hormone in the regulation of muscle PGC-1α and mitochondrial biogenesis.

The Sirtuin family (SIRT 1 to 7) is an NAD-dependent histone/protein deacetylase that interacts with transcription factors and cofactors influencing many metabolic pathways (for review see White & Schenk 2012). SIRT1 deacetylases PGC-1α and thus maintains in its active form capable of binding to chromatin (Gerhart-Hines et al. 2007). Recent studies in cell culture have shown that AMPK allowed to activate SIRT1 and thus deacetylate PGC-1α , by increasing the cellular content of NAD + (Cantó et al. 2009).

Furthermore, it is important to note that the expression of PGC-1α may be regulated by PGC-1α itself *via* its interaction with MEF2 (myocyte enhancer factor-2) and its own promoter region in a loop of autoregulation (Handschin et al. 2003). Such mechanism could contribute to amplify the increase in the expression of PGC-1α when it occurs.

Figure 8. PGC-1α and biogenesis mitochondrial up-streams in skeletal muscle.



2.2. Mitochondria as a source of reactive oxygen species

Originally, it was described that 95-98% of the oxygen is reduced to water at the complex IV of the ETC. However, the transfer of electrons in the ETC is imperfect. In fact, an electron leakage at complex I and III results in 2-5% of cases in the formation of superoxide anion ($O_2^{\bullet-}$) from O_2 which triggers a cascade of ROS production (Chance et al. 1979). However, these values have been recalculated. Brand and colleagues have reassessed the rate of production of ROS by mitochondria and indicated that the upper estimate of the proportion of the electron flow giving rise to ROS was ~0.15%, or ≤10% of the original minimum estimate (St-Pierre et al. 2002). Due to this mechanism, mitochondria are a major cellular source of reactive oxygen species (others sources of ROS will be detailed in a next chapter). To cope with this physiological ROS production, mitochondria have evolved a multileveled defense network comprising detoxifying enzymes and non-enzymatic antioxidants (more detail in Chapter 3).

Under physiological conditions, mitochondrial antioxidant defenses are fully functioning and electron leakage occurs within the physiological range. Thus, oxidative damage is almost completely prevented. In such circumstances, the small amounts generated ROS can act as second messenger molecules that modulate the expression of several genes involved in metabolic regulation and stress resistance (mitochondrial hormesis or mitohormesis; Handy & Loscalzo 2012). Moreover, the small quantities of H_2O_2 and $O_2^{\bullet-}$ generated by the ETC (and by other cellular sources) are essential for force production (Reid et al. 1993). In contrast, excessive ROS generation and/or defective oxidant scavenging can lead to oxidative irreversible damage or essential pathway deregulation which have been implicated in the aging process (Harman 1972; Miquel et al. 1980; Viña et al. 2013) and in the pathogenesis of several conditions, including acute muscle atrophy and sarcopenia as it will be exposed in a next section (Kondo et al. 1994; Reid & Durham 2002; Powers et al. 2011; Handy & Loscalzo 2012).

2.3. The mitochondrial apoptotic machinery

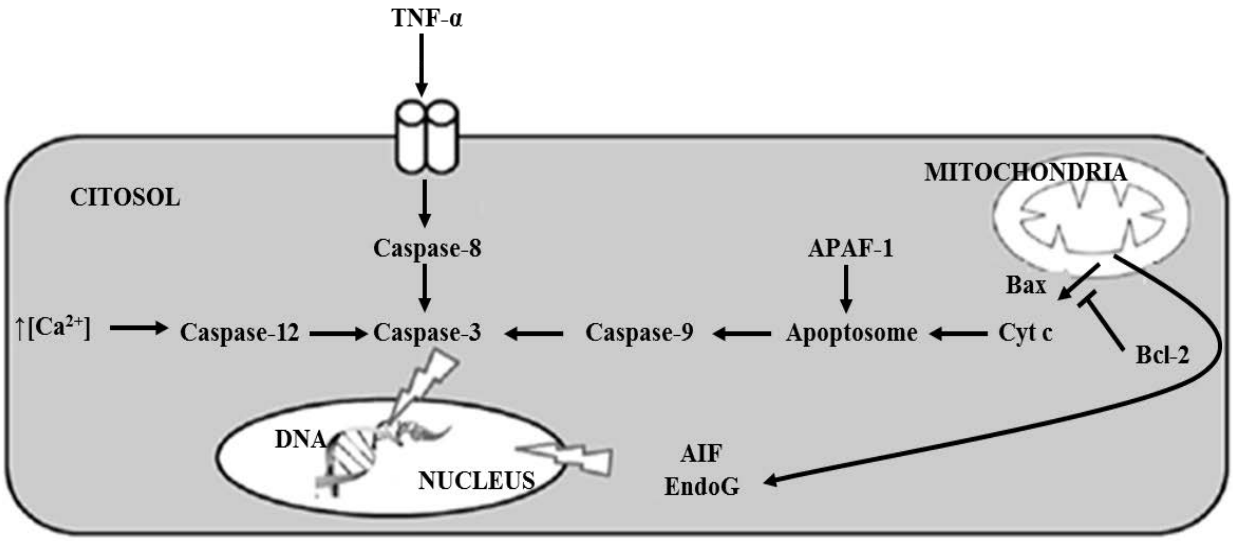
Mitochondria are considered the primary regulator of apoptotic signals and can induce apoptosis through different signaling pathways (Wenz et al. 2009; Marzetti et al. 2013). Apoptosis is a process of programmed cell death which proceeds through a highly coordinated set of morphological and biochemical events, resulting in cellular self-destruction without inflammation or damage to the surrounding tissue (Kerr et al. 1972).

Apoptosis leads progressively to DNA fragmentation, nuclear condensation, proteolysis, membrane deformation and finally to cell fragmentation. This results in apoptotic bodies, which are then supported by macrophages and neighboring cells. As shown in figure 9, apoptosis can be triggered by extrinsic pathway involving the death receptor TNF-α or an intrinsic pathway involving mitochondria. The extrinsic pathway of apoptosis stimulates TNF-α receptor induces the activation of caspase-3 by caspase-8. Mitochondria-induce apoptosis is triggered by two intracellular signaling pathways independent or dependent of caspases (cysteine-dependent aspartate-cleaving proteases) (Danial & Korsmeyer 2004). Caspases exist in the cytoplasm as inactive precursors (procaspases) that can be activated by dimerization or partial degradation. The induction of apoptosis is based on a proteolytic cascade leading to the activation of initiator caspases (i.e. caspase-8, caspase-9, caspase-12) which will itself induce effector caspases (ie caspase-3, caspase-6, caspase-7). The latter then induces DNA fragmentation (*via* caspase-activated DNAase) that leads to cell death. Independent apoptotic caspases pathway operates *via* the mitochondrial release of mediators (e.g. AIF: Apoptosis-Inducing Factor or EndoG: Endonuclease G) capable of inducing DNA fragmentation directly to large scale (see figure 9). *In* addition, opening of the mitochondrial permeability transition pore (mPTP: protein complex comprising the voltage-dependent anion channel (VDAC) in the outer membrane, the adenine nucleotide translocator (ANT) in the inner membrane (IM), and cyclophilin D (CyPD) in the matrix) can induce a sudden increase in membrane permeability, collapse of membrane potential, mitochondrial swelling and rupture of the outer membrane, with subsequent release of death effectors. For instance, following outer membrane permeabilisation, cytochrome c binds Apoptosis Protease-activating factor 1 (Apaf-1) forming an apoptosome leading to caspase-9 activation. Then, this latter activates caspase-3 that finalizes the apoptotic process. Otherwise, it is important to underline the role of Bcl-2 protein family that regulates mitochondrial release of apoptotic mediators mentioned above. Among these proteins, Bcl-2 and Bcl-XL are recognized as anti-apoptotic while Bax, Bak and Bik promote apoptosis. The Bax/Bcl-2 ratio is considered as an index apoptotic status (Marzetti et al. 2010).

Recent studies have shown that PGC-1α could participate in the regulation of apoptotic processes in skeletal muscle. Indeed, isolated mitochondria from muscle of PGC-1α KO mice, exposed to ROS, liberated a greater amount of cytochrome c (indicating increased apoptosis) (Adhihetty et al. 2009). Moreover, chronic overexpression of PGC-1α in muscle tissue (mouse MCK PGC-1α) can effectively prevent the DNA fragmentation associated with

age (Wenz et al. 2009). This anti-apoptotic effect could be explained in part by maintaining the ratio between Bcl-2 and Bax during the aging process (Wenz et al. 2009).

Figure 9. Simplified apoptosis pathway in skeletal muscle (inspired by Marzetti et al. 2012).



2.4. The dynamic nature of mitochondria

When mitochondria are viewed in living cells, it becomes immediately apparent that their morphologies are far from static. Their shapes change continually through the combined actions of fission, fusion and also motility. Fusion and fission events are also crucial for transmitting redox-sensitive signals, maintaining mtDNA integrity, and regulating cell death pathways (for review see Schäfer & Reichert 2009; Youle & van der Bliek 2012).

The balance between fusion and fission is dependent upon a complex mitochondrial dynamics machinery. Mitochondrial fission and fusion processes are both mediated by guanosine triphosphatases (GTPases) in the dynamin family that are well conserved between yeast, flies, and mammals (Hoppins et al. 2007).

Mitochondrial dynamics are centrally involved in the maintenance of cell homeostasis. Indeed investigations utilizing animal knockout models of mitofusion proteins have demonstrated diminished mitochondrial function and biogenesis as well as muscle atrophy (Chen et al. 2010). Conversely, when fusion is no longer possible due to the loss of mitochondrial membrane integrity, fission is responsible for the fragmentation and excision of any altered or damaged mitochondrial components that are subsequently degraded by mitochondrial specific autophagy (i.e. mitophagy as previously described) (Seo et al. 2010).

A functional link therefore appears between mitochondrial dynamics and autophagy, which is essential for mitochondrial homeostasis (Twig et al. 2008). In fact, Parkin and PINK1 (both involved in the regulation of mitochondrial autophagy as previously described) promote mitochondrial fission and inhibit fusion (Deng et al. 2008). The segregation of damaged mitochondria by fission and subsequent inhibition of their fusion machinery are hence prerequisites for their autophagic degradation (Twig et al. 2008).

3. Sarcopenia-related skeletal muscle alterations

As previously described in the first chapter suggested categorization of sarcopenia by EWGSOP, Cruz-Jentoft et al. 2010), sarcopenia can be only age-related (primary sarcopenia) or be the result of others factors such as inactivity, but in both cases, it leads to atrophy at whole muscle level due to changes in both systemic and cellular properties that contribute to loss of organelles, cytoplasmic contents, and proteins from skeletal muscle. The loss of these critical myocyte components results in either fiber atrophy (decrease of the cross sectional area of each fiber) or complete fiber loss (leading to a decrease of the number of muscular fibers), both leading to a decrease in muscle mass. The mechanisms leading to these two phenomena are the same independently the origin of sarcopenia. An imbalance in the protein turnover (Combaret et al. 2009) and an exacerbation of myonuclear apoptosis (Marzetti et al. 2012) are commonly considered as the final cellular mechanisms leading to muscle atrophy in sarcopenia. These latter are themselves dependent on a multitude of systemic and cellular factors (for review see Marzetti et al. 2009; Buford et al. 2010) such as neuromuscular dysfunction (Edström et al. 2007), elevation of oxidative stress (Ji 2001), an increased production of pro-inflammatory cytokines (Lee et al. 2007), insulin resistance (Walrand et al. 2011), a decrease in the production of anabolic hormones (GH, IGF-1, testosterone) (Morley and Malmstrom 2013) and mitochondrial dysfunctions (Calvani et al. 2013). The decrease in capacity of muscle regeneration through satellite cells could be also involved in sarcopenia (Snijders et al. 2009; Hikida 2011).

Muscle atrophy plays a major role in the decrease in muscle strength associated with sarcopenia. However, data from animals showed that the specific strength of isolated muscle fibers (i.e. force normalized to cross sectional area of the fiber) also decreased with age (Renganathan et al. 1998; Thompson & Brown 1999; González et al. 2000; Thompson 2009) but contradictory results have been found in Human (Claflin et al. 2011). Several mechanisms are proposed to explain these results as posttranslational modifications of contractile proteins (Lowe et al. 2001) and/or decoupling of the complex excitation-contraction (Wang et al.

2000). Studies focused on permeabilized muscle fibers have shown that the decrease in the specific strength is also explained by a reduction of the fraction of myosin heads to bind to the actin filaments (Lowe et al. 2001; Lowe et al. 2004). By studying isolated intact muscle fibers, it has been revealed the involvement of excitation-contraction coupling in the changes with age of muscle contractile properties. Thus, the maximum release of calcium from the sarcoplasmic reticulum is reduced in aged rodent muscle tissue (Jiménez-Moreno et al. 2008). This subject being beyond the scope of this work will not be more described.

3.1. Protein turnover alterations

As we discussed in the first chapter, muscle mass decreases with age in human and rodents. Because, the major components of muscle are proteins (after water), and muscle mass is determined by the net relationship between protein synthesis and breakdown, sarcopenia must be due to a relative decrease in protein synthesis, a relative increase in protein degradation, or a combination of both.

3.1.1. Sarcopenia-associated protein synthesis impairment

3.1.1.1. Evidence of a deacreased muscle proteins synthesis during sarcopenia

Data on the effect of aging on whole body protein synthesis are conflicting surely due to different measurement protocols, control of physical activity and diet, correction or not for free fat mass (for review see Nair 1995; Karakelides & Nair 2005; Short et al. 2004). In human, whole body protein synthesis slightly decreases with aging (Balagopal & Rooyackers 1997; Rooyackers et al. 1997; Short et al. 2003; Short et al. 2004) or remains unchanged (Welle et al. 1995; Volpi et al. 2001). It could be explained because the contribution of skeletal muscle to whole body protein synthesis is small (<30%) and consequently slight change in whole body protein does not reflect what is occurring in skeletal muscle.

Thus, it appears more pertinent to study specifically skeletal muscle protein synthesis. The global pool of skeletal muscle protein can be separated into sarcoplasmic, myofibrillar and mitochondrial proteins (these latter decrease with aging and will be specifically studied in a next point). As for whole body protein synthesis, data on muscle protein synthesis in humans are inconsistent with studies reporting a decrease (Balagopal & Rooyackers 1997; Rooyackers et al. 1997; Short et al. 2003) and others no change (Volpi et al. 2001; Hasten et al. 2000; Haddad & Adams 2006). On the other hand, data obtained in rats showed that the protein content of the gastrocnemius decreases during aging (Kimball et al. 2004; Haddad &

Adams 2006). Myofibrillar proteins appear more relevant than the total muscle proteins because of their role in muscle contraction. Data in human agreed on a reduction of their synthesis during aging (Balagopal & Rooyackers 1997; Cuthbertson et al. 2005; Haddad & Adams 2006).

Finally, the study of the synthesis of specific myofibrillar proteins seems the most appropriate approach to linking protein synthesis and sarcopenia and especially myosin heavy chains (total: MHC, or by isoform isoform). Indeed, Balagopal et al (1997) reported that in humans the synthesis of MHC decreases during aging and it is inversely proportional to the concentrations of IGF-1 plasma, strength and muscle mass. These data were subsequently confirmed by Hasten et al (2000). This decrease in the synthesis of MHC is at least due to a decrease in its transcription since the amounts of RNA of different isoforms decrease during aging in particular MHC IIa and MHC IIx isoforms (Balagopal & Schimke 2001; Short et al. 2005). This may explain in part why MHC protein content in muscle of old animals is reduced compared to young animals (Haddad & Adams 2006; Thompson et al. 2006) and why MHC IIa and IIx protein decline by 3 and 1% per decade in humans (Short et al. 2005). This decrease in myofibrillar proteins synthesis appears specific since actin synthesis is not affected by aging in human (Hasten et al. 2000) and its muscle protein content is the same in aged animals (Haddad & Adams 2006; Thompson et al. 2006).

As we discussed in the first part of this chapter, protein synthesis depends on the capacity and efficiency of transcription and translation (RNA synthetizes proteins).

3.1.1.2. Sarcopenia-related molecular alterations leading to muscle protein synthesis decrease

In the muscle cell, the transcriptional ability is reflected by the amount of nuclear DNA which depends on the number of nuclei. Although the amount of DNA (Haddad & Adams 2006; Roberts et al. 2010) and myonuclei number per fiber (Wada et al. 2003; Leeuwenburgh et al. 2005) appear to be stable in aged muscle of rats and humans, the transcriptional efficiency decreases with age as evidenced by the decrease in RNA/DNA ratio (Cuthbertson et al. 2005; Roberts et al. 2010). These results could be explained by a decreased activity of the damaged myonuclei. Indeed, in the soleus muscle of old rats, high levels of apoptosis is observed without elimination of their myonuclei while this elimination happened in the young rats (Leeuwenburgh et al. 2005). The apoptosis process by fragmenting DNA would decrease transcription. On the other hand, as to counteract these phenomena, there is an increase of MRF RNAs such as MyoD, and Myf-5 (Musarò et al. 1995; Alway et al. 2002) but their amount of protein remains unchanged (Kosek et al. 2006).

In resting states, nevertheless muscle type, translational capacity assessed by RNA content in skeletal muscle seems to be the same between adult and old rats (Prod'homme et al. 2005; Haddad & Adams 2006). Similar data in human were recently reported by Roberts et al. (2010). Hence, the 3 aforementioned studies suggest that global translational capacity (i.e. the number of ribosomes) remains intact with mammalian aging in disease-free conditions. However as previously exposed, MHC RNAs decrease in old rats suggesting a specific age-related translational capacity targeting RNAs coding for myofibrillar proteins). On the other hand, it is fairly well established that translational efficiency is decreased with aging in resting in human and rodent. Indeed, RNA/Protein ratio is lower in gastrocnemius of old rats compared to young rats (Haddad & Adams 2006; Prod'homme et al. 2005) and similar data are reported in human muscle biopsies (Cuthbertson et al. 2005; Roberts et al. 2010). These results suggest that younger are seemingly able to translate more muscle protein per unit RNA than older.

The mRNA translation largely controlled by the PI3K/Akt/mTOR pathways is then modulated with aging. This regulation appears to be muscle type- and sexe-dependent (Kimball et al. 2004; Paturi et al. 2010). Here we will focus only on male studies.

Akt, mTOR, p70S6K and their downstreams (rpS6 and eEF2) activation decreased between 6 and 36 months in soleus of male rats which is associated with a weight reduction of the soleus around 40% (Paturi et al. 2010). Activation of Akt and mTOR in EDL continually increased between 6 and 30 months but their downstream rpS6 and eEF2 phosphorylation and the inhibition of 4EBP1 decreased (Paturi et al. 2010) and was associated with a weight reduction of EDL around 30%, suggesting a decreased protein synthesis. Similar results have been reported in various studies in rodents (Parkington et al. 2004; Kimball et al. 2004; Paturi et al. 2010; Rahnert et al. 2011) and in old people (Cuthbertson et al. 2005; Léger et al. 2008) which suggests that an impairment of the PI3K/Akt/mTOR pathways is involved in sarcopenia.

This apparent decrease in the activation of PI3K/Akt/mTOR axis and their downstream could be partially explained by hormonal changes in humans and animals. Indeed, there is decline of GH during aging that is likely secondary to a decrease in pituitary response to hypothalamic growth hormone-releasing hormone (GHRH) and an increase in the inhibitory effect of somatostatin (Kelijman 1991). Moreover, Veldhuis et al. (1995) found a decrease in GH secretory burst amplitude mass with age (maximal rate of GH secretion attained within a release episode). In muscle of elderly subjects, it was reported a decrease in the number of receptors for growth hormone associated with decreased amounts of IGF-1

RNA and decreased IRS-1 and Akt phosphorylation (Léger et al. 2008). Balagopal et al. (1997) showed that lower concentration of plasma IGF-1 is correlated with a decrease in the synthesis of MHC which is itself correlated with a decrease in muscle strength. On the other hand, during aging, there is a decrease in serum and plasma concentrations of testosterone in humans (Balagopal & Rooyackers 1997; Cuthbertson et al. 2005; Léger et al. 2008), which is positively correlated with a decrease in plasma IGF-1 (Balagopal & Rooyackers 1997). Kovacheva et al. (2010) showed that sarcopenic mice have lower testosterone plasma levels compared to young rat associated with a 25-30% decrease of gastrocnemius weight and fast and slow fiber CSA and a lower activation of Akt. Taken together, all these data support the notion that an aging-related decline in IGF-1/Akt/mTOR signaling and net decreases in protein synthesis contribute to sarcopenia.

Moreover, aging is associated with an impaired anabolic response of muscle protein synthesis (for review see Walrand et al. and Rassmussen and Volpi in Cruz-Jentoft 2012). First, postprandial protein synthesis seems particularly decreased in the elderly (Guillet et al. 2004; Cuthbertson et al. 2005) and is one of the mechanisms responsible for the reduction in muscle protein content. Several studies have shown that alterations in postprandial protein synthesis could be explained by the progressive insulin resistance occurring during aging specifically in aged muscle (Guillet et al. 2004; Drummond et al. 2008; Walrand et al. 2008) and also by a lower response to anabolic stimulation by amino acid. These facts are associated with an impaired response of the IGF-1/Akt/mTor pathways. Indeed, following a bolus of essential amino acids, mTOR and p70S6K phosphorylation increased significantly in both young and old people however this phosphorylation increase is lesser in the elderly (Cuthbertson et al. 2005). In response to an infusion of insulin and amino acids, both young and elderly subjects displayed activation of Akt, mTOR, and 4EBP1, but p70S6K activation was not observed in old subjects (Guillet et al. 2004). In addition, moderate chronic inflammatory condition observed in the elderly may also indirectly affect protein synthesis (Balage et al. 2010). This hypothesis is supported by the fact that a clear increase in postprandial protein synthesis is observed in older rodents treated with antioxidants or pharmalogical anti-inflammatory agents (Marzani et al. 2008; Rieu et al. 2009). As it will be exposed in a next chapter, resistance training is one of the best protections against sarcopenia. However, resistance exercise studies typically show an attenuated muscle protein anabolic response in older compared to younger adults (Sheffield-Moore et al. 2005; Kumar et al. 2009; Fry et al. 2011) that are associated with an impaired response of mTOR signaling. Indeed, recently, Fry et al. (2011) and Kumar et al. (2009) showed that after a single bout of

exercise, myofibrillar protein synthesis only increased significantly in young to reach values greater than older people. This was associated with an increased phosphorylation only in the younger group for mTOR, p770S6K and 4E-BP1.

3.1.2. Sarcopenia-associated protein degradation impairment

Studies who had measured protein degradation during aging are still few and the results are contradictory. Nevertheless, several studies in humans and animals have reported a trend to increase (Gaugler et al. 2011; Fry et al. 2013) or an increase (Hasten et al. 2000; Yarasheski 2003) in 3-methylhistidine reflecting increased degradation of myofibrillar proteins. More data are needed to provide a consensus but existing data on the UPS, autophagy and the calcium-activated proteases (i.e. calpain enzymes family and caspase 3) in aged muscle suggest clearly their involvement in sarcopenia.

3.1.2.1. Changes in the calcium-activated proteases

Calpains activity is regulated by several factors, including cytosolic calcium levels and the concentration of the endogenous calpain inhibitor calpastatin (Goll et al. 2003).

In this regard, it is known that aging in skeletal muscle is associated with calcium overload (Fraysse et al. 2006) and calpain activation (Dargelos et al. 2007; Samengo et al. 2012). Indeed, Dargelos et al. (2007) reported a global increase in calcium-dependent proteolytic activity in muscles of aged rats. In such case, protein and RNA levels indicated an up-regulation of calpain 1 expression in muscles of aged rats whereas calpain 2 expression remained unchanged. On the other hand, both calpain 1 and calpain 2 activities increased with aging in skeletal muscle (Dargelos et al. 2007; Samengo et al. 2012). The increased calpain 1 activity is in accordance with its up-regulated expression (Dargelos et al. 2007) whereas calpain 2 activity appear to be up-regulated by another mechanism. In fact, it has been shown *in vitro* that calpain 2 is also modulated by nitric oxide (NO) binding to cysteine in the catalytic domain of the protease, through a process called S-nitrosylation (Koh & Tidball 2000). Recently, Samengo et al. (2012) confirmed this result in mice. Indeed, they showed that during aging there is a decrease in S-nitrosylated calpain associated with a concomitant decrease in neuronal nitric oxide synthase (nNOS) expression (RNA and protein content) leading to an increase in calpain activity associated with a muscle loss in soleus and quadriceps around 20% (Samengo et al. 2012). Contrary to WT mice, mice overexpressing nNOS have both higher calpain S-nitrosylation and nNOS expression, and were totally protected against muscle loss (Samengo et al. 2012).

In accordance with the aged-related increased calpain activity, the activity and/or protein content of calpastatin (endogenous inhibitor of calpains) appear to be decreased in aged muscle rodents (Dargelos et al. 2007; Samengo et al. 2012). On the other hand, calpastatin overexpression in mice inhibits calpain activity in aging skeletal muscle and slows sarcopenia (Samengo et al. 2012).

Several works showed that caspase 3 can promote calpain activation in muscle (Nelson et al. 2012; Samengo et al. 2012) and is involved in diaphragm atrophy during mechanical ventilation (Nelson et al. 2012). Samengo et al. (2012) showed that during aging in parallel to calpain activation there is an increased in caspase 3 activation. Moreover, caspase 3 appears to cleave MHC-IIb. Thus, increase in cleaved MHC-IIb would result of the concomitant action of calpains and caspase 3 and would contribute to explain the lower level of MHC reported in aged skeletal muscle (Haddad & Adams 2006; Thompson et al. 2006; Short et al. 2005). Du et al. (2004) have shown that purified and activated caspase-3 can also cleave actin but the involvement of this mechanism in sarcopenia does not seem to have been studied. On the other hand, Brulé et al. (2010) using a proteomic approach suggested a possible implication of calpains in age-related mitochondria impairment.

3.1.2.2. Changes in the ubiquitin-proteasome system

It has been shown that ubiquitination capacities and ubiquitinated protein content were increased in aged muscle in animals (Altun et al. 2010). As previously stated, proteins ubiquitination is achieved by a complex enzyme system in which the E3-ubiquitin ligases MAFbx and MuRF1 plays an essential role. Interestingly, several studies have observed that MAFbx and MuRF1 RNA levels and protein content increased in muscle of old animals (Clavel et al. 2006; Hepple et al. 2008; Altun et al. 2010). In addition, recent studies have shown that the content of the 26S proteasome was increased in muscle with age in rats (Altun et al. 2010). In humans, the ubiquitin proteasome system appears to function as efficiently in old muscle as it does in adult muscle (Bossola et al. 2008), and aging-related increases in total intramuscular ubiquitin content have been reported (Cai et al. 2004). MAFbx expression seems to be not different between adult and old subjects (Léger et al. 2008; Raue et al. 2007; Whitman et al. 2005), and MuRF1 levels have been reported to either not change (Léger et al. 2008; Whitman et al. 2005) or increase with aging (Raue et al. 2007).

When it is present, the up-regulation of the UPS could be explained by the increase of several of its up-streams. It has been shown in old mice that there was an increase in serum (Carlson, Conboy, et al. 2009) and local intramuscular (Carlson et al. 2008) levels of TGF-β

but no differences in intramuscular myostatin levels were observed (Carlson et al. 2008). Moreover, old mice also display elevated activation of Smad3 (Carlson et al. 2008) and p38 MAPK (Rahnert et al. 2011) (two main pathways downstream of the TGF-β/myostatin receptors). In old people, one study reported an increase in serum levels of TGF-β (Carlson, Conboy, et al. 2009), whereas another showed any difference in circulating levels of myostatin between young and older subjects (Ratkevicius et al. 2011). Moreover, local intramuscular levels of myostatin appear to be elevated with aging in humans (Léger et al. 2008). In addition, the pro-inflammatory cytokines (especially TNF-α and IL-6) recognized to stimulate muscle proteolysis *via* the UPS (Llovera et al. 1997) increased concentration in muscle during aging (Schaap et al. 2009).

As well as protein synthesis, UPS responses to catabolic or anabolic stimuli seem to be impaired during aging. Combaret et al. (2005) reported in aged rat skeletal muscle a lack of postprandial inhibition of proteasome-dependent proteolysis that can be restored by leucine-supplemented diet. Following a single bout of resistance exercise, for both adult and old subjects there was an increase in MuRF1, but only the old subjects had an increase in MAFbx (Raue et al. 2007). Others studies reported contrary data (Haddad & Adams 2006; Léger et al. 2008; Fry et al. 2013).

3.1.2.3. Impaired autophagy

Although surprising, almost all studies seem to agree on a reduction in protein degradation *via* autophagy in muscle aging and sarcopenia in humans and animals (McMullen et al. 2009; Wohlgemuth et al. 2010; O'Leary et al. 2013; Fry et al. 2013; Kim et al. 2013).

Despite these findings, the different steps of the latter would not be affected in the same way. Indeed, the increased Beclin 1 protein content observed in aged muscle in elderly humans (Fry et al. 2013) or in very old animals (Wohlgemuth et al. 2010; O'Leary et al. 2013) suggests an increase in the induction of autophagy. However, autophagosome formation appears impaired in skeletal muscle during aging as almost all the studies showed a down-regulation of LC3-1 and 2 or a decrease of LC3-2/LC3-1 ratio in skeletal muscle (McMullen et al. 2009; Wohlgemuth et al. 2010; Fry et al. 2013; Kim et al. 2013). In the same way, decreased RNA level (Wohlgemuth et al. 2010) and protein content (Kim et al. 2013) of Lamp-2 in aged muscle suggest a delayed fusion of autophagic vacuoles with lysosomes. Ultimately, lysosomes exhibit accumulation of lipofuscin deposits during aging, increasing the size of these organelles but decreasing their functionality (Cuervo & Dice 2000; O'Leary

et al. 2013). Thus, the last step of autophagy is impaired during aging leading to an accumulation of protein aggregates and damaged mitochondria (Terman & Brunk 2006).

3.2. Mitochondria dysfunctions and sarcopenia

Given the vital functions carried out by mitochondria in the context of energy provision, redox homeostasis, and regulation of several catabolic and cell death pathways it is not surprising that age-related alterations of mitochondrial functions are placed at the center of sarcopenia by numerous authors (for review see Marzetti et al. 2013; Konopka & Sreekumaran Nair 2013; Calvani et al. 2013). One major consequence of the age-associated mitochondrial dysfunction is a decline in bioenergetics that is witnessed by a decrease in both resting and maximal oxygen consumption ($\dot{V}O_{2max}$) with advancing age in humans (Short et al. 2004) and mice (Lee et al. 2010) and by a decreased endurance capacity in old rats compared to young rats (Derbré et al. 2012). Moreover, perturbations in skeletal muscle mitochondrial energetics have been correlated with reduced $\dot{V}O_{2max}$ (Short et al. 2005), walking capacity (Coen et al. 2013) and maximal isometric strength (Safdar, Hamadeh, et al. 2010) in older adults and are associated with an increase in muscle fatigability in old rats (Chabi et al. 2008). The bioenergetic failure of the aged muscle is associated with a reduction in mitochondrial abundance and function.

3.2.1. Reduced mitochondrial content and function with age

Numerous studies in human and rodent have revealed age-related declines in mitochondrial mass.

Studies revealed lower mitochondrial volume density in older adults (Conley et al., 2000) and in senescent rodent (Lee et al. 2010; O'Leary et al. 2013). A decline in mitochondrial content, as represented by mitochondrial DNA (mtDNA) copy number, has also been demonstrated in rodents (Ibebunjo et al. 2013) and humans (Welle et al. 2003; Short et al. 2005). Muscle mtDNA age-related decrease is associated with a concomitant decrease in muscle concentrations of mRNAs encoded by the latter (Welle et al. 2003; Short et al. 2005) and reduced levels of mitochondrial protein synthesis (Rooyackers et al., 1996). Finally, expression and/or maximal activities of proteins involved in Krebs cycle (i.e. citrate synthase; CS) and/or proteins involved in the ETC (cytochrome c oxidase; COX) are decreased with advancing age and numerous studies used these markers to assess a decreased mitochondrial content in older humans (Rooyackers et al. 1996; Safdar, Hamadeh, et al. 2010), monkeys (Lee et al. 1998; Pugh et al. 2013) and rodents (Chabi et al. 2008; Ibebunjo et al. 2013;

O'Leary et al. 2013). These findings are expected to alter mitochondrial function. Indeed, studies revealed a decline in maximal mitochondrial ATP synthesis (Short et al. 2005; Lee et al. 2010), O_2 consumption (Lee et al. 2010; Joseph et al. 2013; Coen et al. 2013), oxidative phosphorylation (OXPHOS) activity (Conley et al. 2000). Collectively, reductions in mitochondrial proteins and volume may limit ATP production for energy demanding processes such as myocellular remodeling to maintain protein quality which is reflected by the concomitant decrease in whole-body bioenergetics and muscle protein anabolism over the course of aging (Balagopal & Rooyackers 1997; Short et al. 2004).

The reduction in mitochondrial abundance and function with advancing age is likely the result of a vicious cycle involving oxidant production and damage/depletion of mitochondrial DNA (mtDNA), and defective mitochondria quality control (Marzetti et al. 2013).

3.2.2. The vicious cycle between oxidative stress and mitochondrial dysfunction in the aged muscle

As well reviewed by Gomez-Cabrera et al. (2012) mitochondria are sources and targets of damage during aging in several tissues including skeletal muscle. The mtDNA is intrinsically vulnerable to oxidative damage due to its proximity to the source of oxidants, the absence of histones and introns, and a less robust repair system compared with nuclear DNA (Yakes & Van Houten 1997; Wei & Lee 2002). Mitochondrial dysfunction arising from oxidative damage to mtDNA would trigger a vicious cycle in which the synthesis of defective ETC subunits, results in mitochondrial OXPHOS impairment, decreased ATP production and further ROS generation (Harman 1972; Miquel et al. 1980). During aging there is a concomitant increased ROS production (Chabi et al. 2008) and decreased antioxidant defenses in skeletal muscle mitochondria (Safdar, Hamadeh, et al. 2010). These results could explain the increased oxidative damage to mitochondrial lipids (Braga et al. 2008; Wohlgemuth et al. 2010), proteins (Figueiredo et al. 2009; Lee et al. 2010) and overall to mtDNA (Short et al. 2005; Lee et al. 2010) observed in muscles from aged rodents and humans. Moreover, numerous studies observed an age-related accumulation of mtDNA mutations in skeletal muscle in various species due to oxidative stress (Lee et al. 1998; Bua et al. 2006; Figueiredo et al. 2009; Lee et al. 2010). These mtDNA mutations lead to ETC abnormalities associated with morphological aberrations of muscle fibers (Bua et al. 2006).Conversely, mtDNA mutations and ETC abnormalities were absent in phenotypically normal regions within individual fibers. Finally, concomitant oxidative mtDNA damage,

mtDNA mutations and mitochondrial dysfunctions (e.g. decreased ATP production and O_2 consumption) have been detected in muscles from aged rodent, primates and human (Lee et al. 1998; Bua et al. 2006; Figueiredo et al. 2009; Lee et al. 2010). These studies support the hypothesis that oxidative damage and mtDNA mutations contribute to muscle aging and the diseases associated with advancing age.

However, the degree of involvement of mtDNA mutations in mitochondrial dysfunction in the elderly muscle remains controversial. Indeed, it has been shown that these dysfunctions appear with age before mtDNA is necessarily affected (Conley et al. 2007). This would imply other factors responsible for mitochondrial dysfunction such as dysregulation of mitochondrial biogenesis and quality control of mitochondrial proteins.

3.2.3. Possible involvement of mitochondria dynamics in sarcopenia

The morphology and mitochondrial function depend directly on the balance between the synthesis and assembly of mitochondrial proteins, and removal of those damaged or improperly assembled.

Mitochondrial renewal is performed by the degradation of dysfunctional or unnecessary mitochondria through mitophagy and the synthesis of new organelles *via* biogenesis. Accumulating evidence indicates that mitochondrial turnover is altered during muscle aging, potentially affecting mitochondrial function and myocyte homeostasis (for review see Viña et al. 2009; Calvani et al. 2013). Indeed, as previously presented, there is a decrease in the synthesis of mitochondrial proteins (Rooyackers et al. 1996). Numerous studies have detected a decrease of compounds (i.e. PGC-1α, NRF-1) of the mitochondriogenesis cascade in skeletal muscle during aging in old rodents and monkeys (Chabi et al. 2008; Derbré et al. 2012; Koltai et al. 2012; Ibebunjo et al. 2013; Pugh et al. 2013) and elderly people (Safdar, Hamadeh, et al. 2010). In most studies, the decreased expression of PGC is associated with decreased expression and/or activity of some of its activators as AMPK (Lee et al. 2010; Koltai et al. 2012; Pugh et al. 2013), NAMPT (Pugh et al. 2013) and Sirt 1 (Joseph et al. 2013). Moreover, these data are supported by an overall reduction in genes encoding proteins of the ETC in muscle tissue (Ibebunjo et al. 2013).

On the other hand, a reduced ability of degradation pathways to remove whole or damaged compartments of mitochondria could lead to impaired organelle bioenergetics (Huang & Hood 2009). The major pathways that contribute to mitochondrial protein quality control include intra-mitochondrial proteases and autophagy. Studies have illustrated that with increasing age, the activity and expression of the intra-mitochondrial Lon protease is reduced,

reflected by an accumulation of dysfunctional aconitase (Bota et al. 2002; Bota & Davies 2002; Koltai et al. 2012). Otherwise, dysfunctional mitochondria are normally eliminated by mitophagy (autophagy of mitochondria). As previously described, autophagy seems to be less effective with advancing age, especially in muscle tissue mainly because of alterations in autolysosome formation and the function of lysosomes. However, O'Leary et al. (2013) observed an increase of the localization of p62 and Parkin (two specific markers of mitophagy) with mitochondria during aging suggesting a more active mitophagy during its first steps. It could be an attempt to remove giant, dysfunctional mitochondria, characterized by highly interconnected networks and ultrastructural abnormalities which are frequently encountered in aging muscles (Beregi & Regius 1987).

These giant mitochondria could be due to altered mitochondrial degradation associated with an imbalance in mitochondrial fusion-fission events (Calvani et al. 2013). The data concerning the involvement of fusion-fission cycle in sarcopenia are still few and contradictory. Indeed, some studies have reported a decrease in gene expression and amounts of RNA encoding markers of both fusion (i.e. Mfn 1, Mfn 2 and Opa 1) and fission (i.e. Fis 1 and Drp 1) in elderly muscle (Crane et al. 2010; Ibebunjo et al. 2013). In contrast recently, Iqbal et al (2013) reported an increase in protein content of Fis 1 and Drp 1 with a parallel decreased in protein expression of Mfn 2. Finally, two studies (O'Leary et al. 2013; Koltai et al. 2012) reported a concomitant increase in the protein content of markers of fusion (Mfn 1, Mfn 2 and Opa1) and fission (Fis 1). These data suggest that the cycle of fission and fusion may be elevated with age which could lead to the formation of giant dysfunctional mitochondria as previously exposed. The phenomenon is proposed to be an attempt to fight against mtDNA mutation and oxidative damage (Calvani et al. 2013).

More studies are needed to bring a conclusion on the role of mitophagy and mitochondria fusion-fission cycle in muscle aging.

3.2.4. Mitochondria-mediated apoptosis in sarcopenia

A relevant consequence of mitochondrial dysfunction is the activation of apoptosis, a mechanism believed to represent a final pathway through which sarcopenia ensues (for review see Marzetti et al. 2010; Marzetti et al. 2013; Calvani et al. 2013). This idea is supported by the observation that mitochondrial apoptotic signaling correlates with slow walking speed and reduced muscle volume in older persons (Marzetti, Lees, et al. 2012). Moreover numerous studies showed that extent of apoptotic DNA fragmentation increases in skeletal muscle over the course of aging paralleling the development of sarcopenia (Siu et al. 2006; Braga et al.

2008; Marzetti, Wohlgemuth, et al. 2008; Wohlgemuth et al. 2010; Kovacheva et al. 2010). As previously exposed, mitochondria-driven apoptosis can proceed with or without the participation of caspases. Studies indicate that both pathways are involved in aging muscle (for review see Marzetti et al. 2013; Calvani et al. 2013).

Numerous studies have shown in aged rodent muscle an imbalance between pro- and anti-apoptotic members of the Bcl-2 family proteins that favor outer mitochondrial membrane (OMM) permeabilization. Indeed, increased expression of Bax and reduce levels of Bcl-2 have been detected in skeletal muscle of old rodents with a dramatic decrease of the Bax/Bcl-2 ratio, usually used of an apoptotic index (W. Song et al. 2005). However several studies reported a concomitant up-regulation of both Bax and Bcl-2 (Pistilli et al. 2006; Marzetti, Wohlgemuth, et al. 2008) with no change in their ratio (Marzetti, Wohlgemuth, et al. 2008). However, the lack of change in the Bax/Bcl-2 ratio should not be interpreted as a lack of change in apoptosis. In fact, Braga et al. (2008) demonstrated in gastrocnemius of old mice an increased expression of Bcl-2 with a parallel increase in its phosphorylated form that is associated with its inactivation and consequently inhibition of its anti-apoptotic action. An enhanced susceptibility towards opening of the mitochondrial permeability transition pore (mPTP) has also been observed in skeletal muscle of old rats (Chabi et al. 2008). Opening mPTP would be associated with release of apoptotic factor as shown by the reported cytosolic cytochrome c (Siu et al. 2005), APAF-1 (Dirks & Leeuwenburgh 2004; Siu et al. 2005) and the active form of caspase-9 (Tamilselvan et al. 2007; Braga et al. 2008) protein content increase in aged muscle tissue. Moreover, Wohlgemuth et al. (2010) found increased caspase-3 and caspase-9 activities in skeletal muscle of old rat. These data support an activation of caspase-dependent pathway in aged muscle tissue. Although, other studies observed no change in cytosolic cytochrome c content in these tissue (Dirks & Leeuwenburgh 2004; Siu et al. 2006; Marzetti, Wohlgemuth, et al. 2008). These controversial results regarding the dependent caspase pathway suggest that the signaling pathway independent of caspases also contributes to the exacerbation of apoptosis in sarcopenic muscle. Indeed, it has been shown that advancing age was associated with increased protein content of nuclear factor EndoG and AIF within muscle tissue (Dirks & Leeuwenburgh 2004; Marzetti, Wohlgemuth, et al. 2008). Furthermore, Chabi et al. (2008) showed an increase in the release of EndoG factor in mitochondria extracted from older muscle tissue compared with mitochondria from young muscles. In addition, the negative correlation reported by Marzetti et al. (2008) between nuclear expression of these two factors and muscle mass suggests the involvement of caspase-independent pathway in sarcopenia.

Finally, apoptosis leads to DNA fragmentation which could explain the age-related decrease in transcriptional efficiency as previously exposed. As muscle fibers are post-mitotic cells, raising DNA content can only come from the incorporation of new nuclei of external origin, most often from satellite cells (SC) (Adams 2006). Therefore, a sufficient pool of functional satellite cells would be essential to continuously maintain the functional DNA amount in muscle fibers but during aging their number and capacity to proliferate appear to be decreased.

3.3. Satellite cells impairment

Studies in rodents have shown that aging is associated with a reduction in the number of quiescent and activated SC within muscle tissue (Dedkov et al. 2003; Brack et al. 2005).

In humans, it appears that the SC pool is maintained until around 70 years (Roth et al. 2000; Dreyer et al. 2006; Petrella et al. 2008) and then declines (Renault et al. 2002; Kadi et al. 2004; Sajko et al. 2004; Verdijk et al. 2007). This reduction also seems to occur preferentially in type II muscle fibers (Verdijk et al. (2007) which coincides exactly with the preferential development of sarcopenia in the fast muscle type.

It is also important to note that advancing age is associated with an impaired SC proliferation capacity (Conboy et al. 2003; Matsuba et al. 2009). Replicative senescence is a mechanism often invoked to explain this phenomenon (Jejurikar & Kuzon 2003). However, this mechanism does not seem relevant to sarcopenia since the *in vitro* proliferation potential of SC remains broadly unchanged with age (Renault et al. 2000). However, the SC isolated from muscle men or older rodents present alterations in their differentiation capacity leading to the formation of less structured and more fragile myotubes (Renault et al. 2000; Lees et al. 2006).

SC extrinsic factors such cellular or systemic environment are certainly partly responsible for the deterioration of the regenerative capacity of aged muscle. Indeed, it has been shown that older SC are able to produce an effective regenerative response when exposed to a young systemic environment or cell (Carlson & Faulkner 1989; Conboy et al. 2005; Carlson & Conboy 2007). Recognized as activating the SC, the Notch signaling pathway may play a key role in this phenomenon (Carlson & Conboy 2007). A down regulation of this pathway has been reported by Kovacheva et al. (2010) in gastrocnemius of sarcopenic rat. In the same study, a treatment with testosterone (known to decrease during aging) up-regulated this pathway. Moreover, using heterochronic parabiosis (two animals of different ages are joined to test for systemic regulators of aspects of aging), it has been

demonstrated that Notch (known to regulate satellite cells activation) is impaired in aged muscle tissue but restored in a young systemic environment (Conboy et al. 2003; Carlson, Suetta, et al. 2009).

Oxidative stress may be also responsible for reducing the activity of SC in aged muscle tissue (see next chapter about oxidative stress and sarcopenia). Moreover, myostatin is recognized as an inhibitor of muscle regeneration. Some studies showed that myostatin decreases myoblast differentiation but also inhibits their proliferation through modulation of specific inhibitors of cyclin-dependent kinases activity (CKI, i.e. p21 and p27) (Thomas et al. 2000; Langley et al. 2002). Increased intramuscular levels of myostatin reported in elderly people (Léger et al. 2008) and old rats (Kovacheva et al. 2010) could participate to the decrease activity of SC.

4. Chapter 2 abstract

The loss of muscle strength in sarcopenia observed is primarily due to muscle atrophy, while a decrease in the specific strength is also involved.

Muscle atrophy is explained by the reduction of myofibrillar and mitochondrial proteins synthesis, and their increased proteolysis *via* the ubiquitin-proteasome system and the calcium-dependent activation of proteases (i.e. calpains and caspases). The decrease in mitochondrial dynamics (biogenesis *vs* degradation *via* autophagy, fusion and fission) leads to the accumulation of defective mitochondria, which then fall into a vicious circle, in which RONS production increases. This RONS overproduction entails mtDNA mutations, which in turn lead to defective ETC protein synthesis and ultimately result a decreased ATP production. Furthermore, exacerbation of apoptosis results in an increase of the DNA fragmentation which could decrease transcriptional efficiency. This phenomenon is probably worsened by alterations in muscle regeneration capacity limiting the incorporation of new nuclei in aging muscle fibers. Reducing the pool of satellite cells and their capacity for proliferation and differentiation (in particular due to a less functional cellular and systemic environment) appear to be responsible for muscle regeneration alterations.

All these mechanisms contribute to the onset of sarcopenia and are controlled by numerous signals as decreased production of anabolic hormones (GH, IGF-1, testosterone, insulin). Links and interactions between these multiple factors remain partly unknown. A potential candidate could be chronic oxidative stress, whose recent studies emphasize its involvement in sarcopenia.

Chapter 3: The contribution of oxidative stress to sarcopenia

1. Generalities on oxidative stress

1.1. Definitions

Within the organism, predominately oxygen or nitrogen molecules, electronically unstable, are rapidly converted into reactive species derived from of oxygen (ROS) or nitrogen (RNS). ROS and RNS (RONS) may be free radical (e.g. superoxide radical $O_2^{-\bullet}$, nitric oxide $NO^{\bullet}$) or non-radical species (e.g. hydrogen peroxide H_2O_2). In this work when it is not specified free radicals are assimilated to all reactive species (free radical and non-radical reactive species derived from O_2 and N).Free radical species are molecules or molecule fragments, containing one or more unpaired electrons in their outer orbital. Due to their high reactivity, RONS often have an average lifespan very short from a billionth of a second (e.g. 10^{-9}s for the hydroxyl radical $OH^{\bullet}$) to few seconds (e.g. 3-5s for the nitric oxide $NO^{\bullet}$)

Under physiological conditions, RONS are continuously produced in small quantities in the body. Nevertheless, this basal production is effectively controlled by the antioxidant defenses (defined as any substance that, when presents at low concentrations compared to oxidizable substrate, significantly reduces or inhibits oxidation of the substrate, Halliwell & Gutteridge 1995) which can prevent the formation of new RONS (primary antioxidant or enzymatic antioxidants), direct scavenge RONS when primary systems are overwhelmed (secondary antioxidants or non-enzymatic antioxidants) or repair damaged biomolecules (tertiary antioxidant), thus limiting oxidative damage. In this case, there is equilibrium in the "oxidants-antioxidants" balance. However, if antioxidant defenses are no longer able to support the production of RONS, an overproduction of reactive species in the cell occurs causing irreversible damage and disrupting intracellular signaling pathways. Then, there is an imbalance of the "oxidants-antioxidants" balance leading to an impaired redox homeostasis. This phenomenon is commonly called oxidative stress (OS) (Sies 1985; Jones 2006).

RONS are toxic and are considered responsible for the oxidative damage of biological macromolecules such as nucleic acids (e.g. nuclear and mitochondrial DNA and RNA), lipids (e.g. lipid membranes and circulatory lipids), proteins (e.g. structural and regulatory) and carbohydrates. These oxidative damage may be the source of cellular dysfunctions.

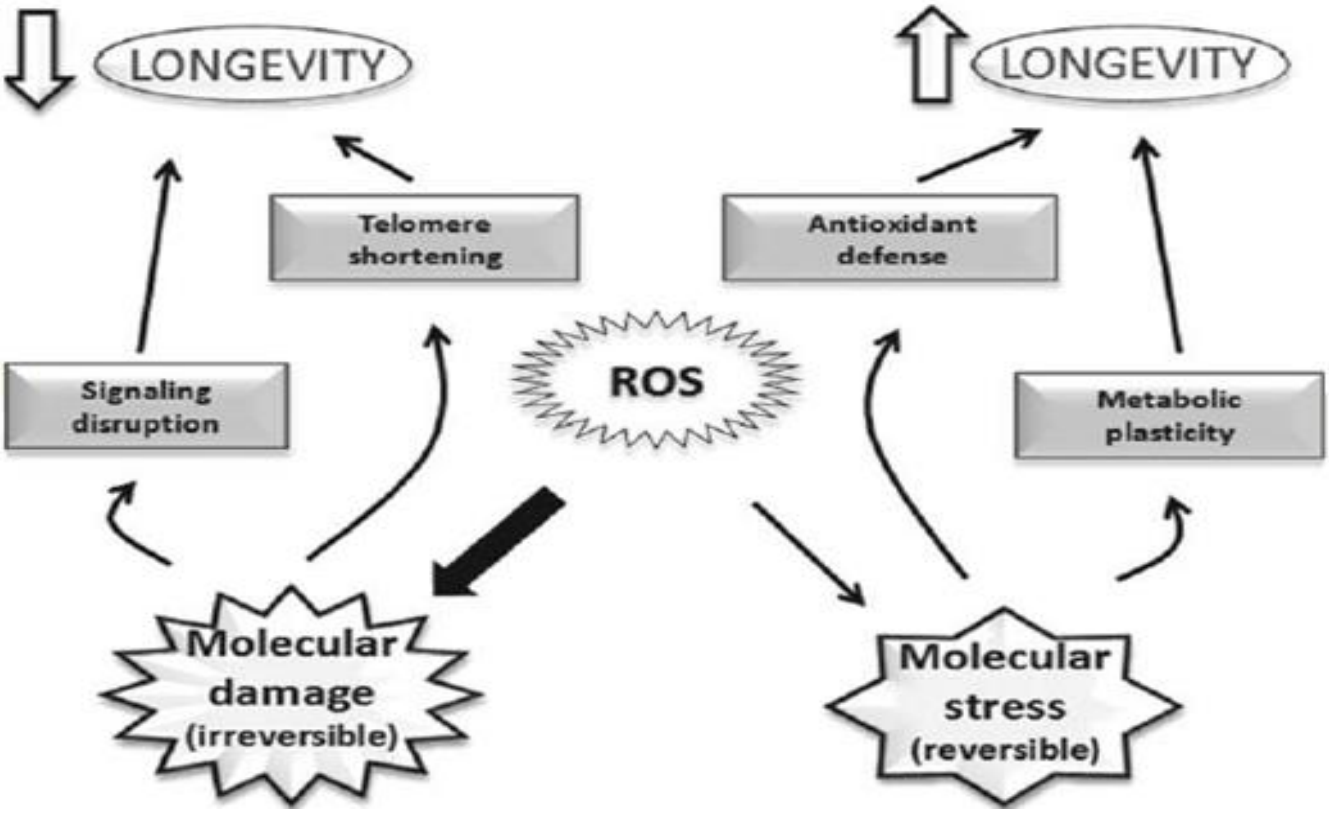
1.2. Theories of aging related to oxidative stress

More than three hundred theories of aging have been postulated (for review see Medvedev 1990). One of the most prominent theories to explain aging is the free radical theory of aging which was initially proposed by Harman during the 1950s (HARMAN 1956). It proposes that free radicals derived from oxygen are responsible for damage associated with aging. The antioxidant systems are unable to counterbalance all the free radicals continuously generated during the life of the cell. This results in oxidative damage in the cell and thus in tissues. Throughout the discoveries of the past 60 years, the free radical theory of aging has evolved until its last versions (for review see Viña et al. 2007; Viña et al. 2013).

As previously described, the transfer of electrons in the ETC is imperfect and an electron leakage at complex I and III results in the formation of superoxide anion ($O_2^{\bullet-}$) from O_2 which triggers a cascade of RONS production (Chance et al. 1979; St-Pierre et al. 2002). Due to this mechanism, mitochondria are a major cellular source of reactive oxygen species. Harman first suggested that mitochondria are key organelles involved in aging (Harman 1972). However, Miquel and co-workers proposed in 1980 the mitochondrial theory of free radicals in aging (MFRTA) (Miquel et al. 1980). The theory suggests that senescence is the result of damage caused by ROS to the mitochondrial genome in post mitotic cells (Miquel et al. 1980). Mitochondria from post mitotic cells use oxygen at high speeds, thus producing RONS which cause OS as they overwhelm the antioxidant cellular defenses (Miquel & Fleming 1986).

Throughout the discoveries of the past 30 years, the free radical of aging (assimilated to the MFRTA) has suffered a lot due to studies in favor or against it (for review see Viña et al. 2013) and recently two new versions have been stated : the redox stress theory of aging (Sohal & Orr 2012) and the cell signaling disruption theory of aging (Viña et al. 2013). The redox stress theory of aging proposes that aging-associated functional losses are primarily caused by a progressive pro-oxidizing shift in the redox state of the cells, which leads to the overoxidation of redox-sensitive thiols proteins and the consequent disruption of the redox-regulated signaling mechanisms. The cell signaling disruption theory of aging (see figure 10) is based on the double edge sword of free radicals and their hormetic effects. In fact when the aggression by free radicals is mild, a stress is caused leading to reversible oxidative damage and this may have signaling effects leading to an up-regulation of antioxidant defenses and metabolic plasticity that would promote longevity and health. However, when radicals cause severe damage on biomolecules it causes irreversible alterations leading to disruption signaling and telomeres shortening that would promote disease and death (Viña et al. 2013).

Figure 10. The cell signaling disruption theory of aging (extracted from Viña et al. 2013).



2. Oxidative stress in sarcopenic skeletal muscle

2.1. Increased RONS production in skeletal muscle is associated with sarcopenia

Both in humans and animals, numerous studies showed that sarcopenic muscle exhibits an increased RONS production (e.g. $O_2^{\bullet-}$ et H_2O_2) (Capel et al. 2004; Capel, Rimbert, et al. 2005; Capel, Demaison, et al. 2005; Chabi et al. 2008; Jackson et al. 2011; Andersson et al. 2011; Miller et al. 2012) and content (Andersson et al. 2011; Janna R. Jackson et al. 2010; Jackson et al. 2011; Ryan et al. 2011; Sullivan-Gunn & Lewandowski 2013) (see table 9). There are numerous RONS generation sources in skeletal muscle that are resumed in the figure 11. As it will be exposed in this section, studies have shown the involvement of some sources in the increased production of RONS during sarcopenia (e.g. mitochondria, xanthine oxidase...).

2.1.1. Mitochondria as sources of RONS

Superoxide anion generation by adding an electron in the last electronic layer of oxygen, may result from a number of intracellular sources (see figure 11). In basal state, mitochondria are usually considered as the main $O_2^{\bullet-}$ source of production in muscle tissue. Indeed, 2-5% of the total oxygen consumed by mitochondria is subjected to a mono-electronic reduction leading to $O_2^{\bullet-}$ production (Boveris & Chance 1973). Then, $O_2^{\bullet-}$ may leads to the formation of many RONS within the mitochondrial matrix (see figure 11) including especially in this case H_2O_2 under the action of manganese-dependent superoxide dismutase (MnSOD) which is mainly present in mitochondria (Boveris & Chance 1973).Then, H_2O_2 is detoxified into O_2 and H_2O by glutathione peroxidase 1 (Gpx-1) and peroxiredoxine III. Alternatively, $O_2^{\bullet-}$ can be released in the intermembrane space (IMS) where it is converted to H_2O_2 by copper-zinc-dependent SOD (ZnSOD). In addition, $O_2^{\bullet-}$ leaked into the IMS can be scavenged by cytochrome c (Pasdois et al. 2011). Numerous studies in human and animals have shown an increased production of ROS (i.e. $O_2^{\bullet-}$ and H_2O_2) and RNS by mitochondria during aging associated with a decreased in muscle mass, muscle function and physical capacity (Capel et al. 2004; Capel, Rimbert, et al. 2005; Chabi et al. 2008; Ryan et al. 2011; Andersson et al. 2011; Miller et al. 2012) (see table 9). Mitochondrial overproduction of $O_2^{\bullet-}$ observed with advancing age can be explained mainly by dysfunction of the ETC (Ji 2001). The complex I (NADH-ubiquinone reductase) and III (ubiquinone-cytochrome c reductase) might be the cause of the mitochondrial overproduction ROS with advancing age (Capel et al. 2004; Capel, Rimbert, et al. 2005; Chabi et al. 2008) (see figure 11). Parallel to this mitochondrial overproduction of $O_2^{\bullet-}$ studies observed an increase in the Mn-SOD activity (Ji et al. 1990; Jackson et al. 2011; Ryan et al. 2011) which would explain the observed concomitant H_2O_2 mitochondrial overproduction (Capel et al. 2004; Capel, Rimbert, et al. 2005; Miller et al. 2012) According to the MRFRA, RONS production during the life would lead to an accumulation of oxidative damage to the mitochondrial compounds especially to mtDNA leading to mtDNA mutations. These mutations would lead to the synthesis of defective ETC subunits which would result in an increase of RONS leakage leading to further oxidative damage.

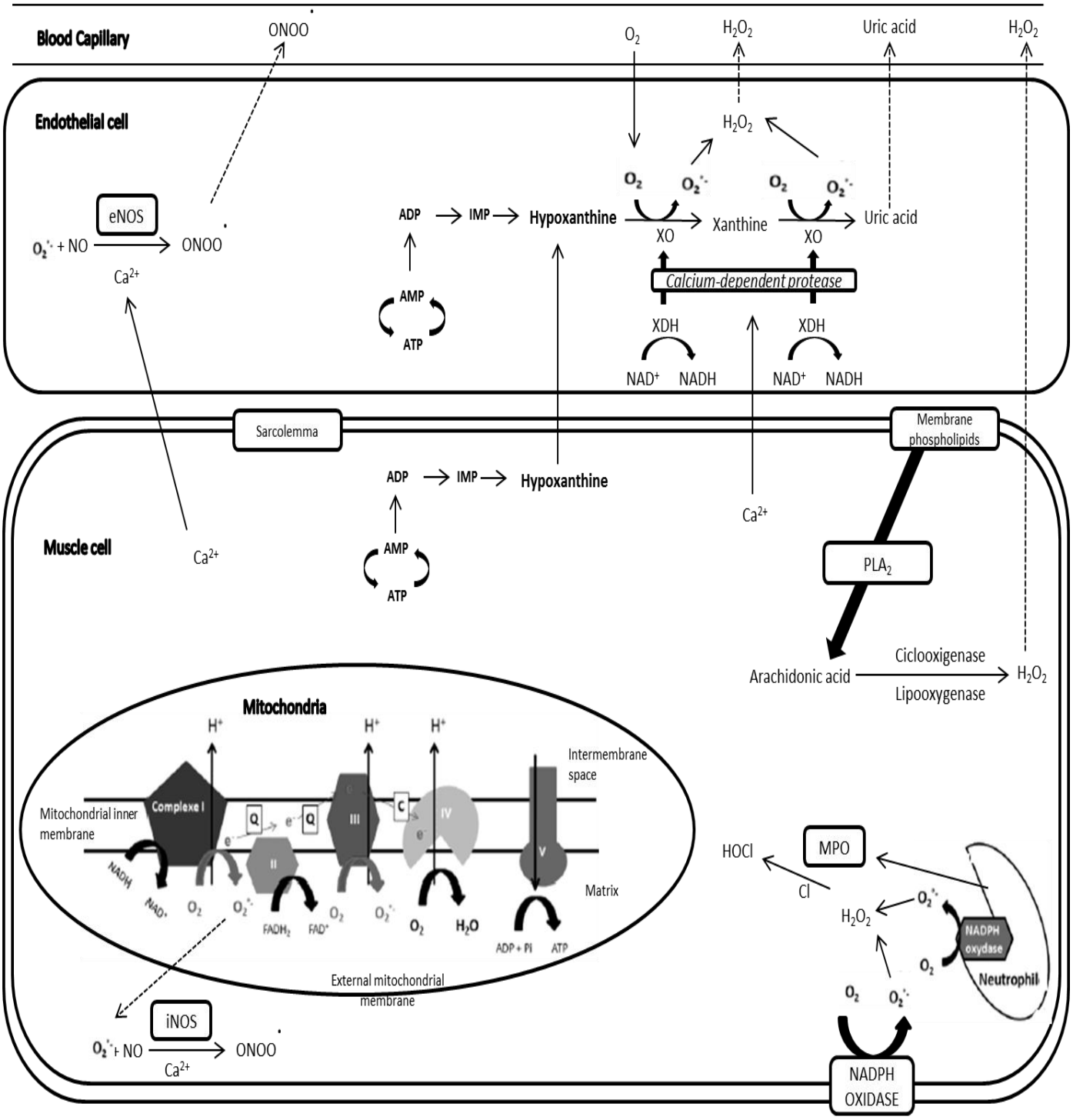
In humans and rodents muscles, numerous studies have shown that these increased oxidative damage impair mitochondrial lipids (Braga et al. 2008; Wohlgemuth et al. 2010), proteins (Figueiredo et al. 2009; Lee et al. 2010) and overall mtDNA (Short et al. 2005; Lee et al. 2010). Moreover, increase in mitochondrial Ca^{2+} concentration observed during aging would result in disruption of mitochondrial membrane potential, which could be involved in

the increased production of RONS by mitochondria. The increase in mitochondrial ROS production with aging occurs mainly in the type I muscles as the soleus of older rats (Capel et al. 2004). However, it appears that the basal mitochondrial ROS production is markedly higher in predominantly glycolytic muscles, regardless of age (Capel et al. 2004; Anderson & Neufer 2006).

Table 9. Sarcopenia-associated mitochondria RONS production.

Study	Specy	Age	Mitochondria RONS production	Skeletal muscle RONS content	Muscle mass and/or function impairment	Physical capacity impairment
Capel et al. 2004	Rats	4 m *vs* 24 m	ROS (H_2O_2)		Muscle weight decrease (Tibialis anterior)	
Capel, Demaison, et al. 2005	Rats	Adult *vs* 21 m	ROS (H_2O_2)		Muscle weight decrease (gastrocnemius)	
Capel, Rimbert, et al. 2005	Human	23 y *vs* 67 y	ROS (H_2O_2)			VO2max decrease
Chabi et al. 2008	Rats	6 m *vs* 36 m	ROS		Muscle weight and strength decrease (soleus,tibialis anterior, plantaris), fatigability increase	
Jackson et al. 2010	Rats	6 m *vs* 34 m		ROS (H_2O_2)	Muscle weight decrease (gastrocnemius)	
Ryan et al. 2011	Mice	3-5 m *vs* 26-28 m	ROS ($O_2^{\bullet-}$, $OH^{\bullet}$)	ROS (H_2O_2)	Strenght decrease	
Jackson et al. 2011	Mice	6 m *vs* 18 m		ROS (H_2O_2)	Muscle weight decrease (gastrocnemius, plantaris)	
Andersson et al. 2011	Rats	3-6 m *vs* 24 m	ROS and RNS	ROS	Muscle weight and strength decrease (EDL)	
Miller et al. 2012	Mice	2 m *vs* > 24 m	ROS ($O_2^{\bullet-}$,H_2O_2)		Muscle atrophy	
Sullivan-Gunn & Lewandowski 2013	Mice	6 m *vs* 18 m and 24 m		ROS ($O_2^{\bullet-}$,H_2O_2)	Muscle atrophy	

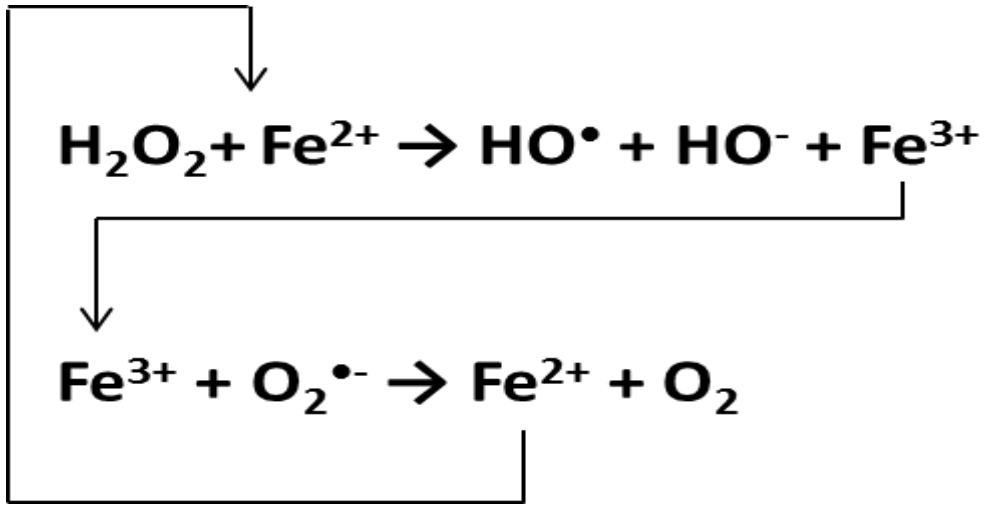
Figure 11. Potential free radicals productions sites in skeletal muscle during sarcopenia.



2.1.2. Free iron accumulation is associated with sarcopenia

Fenton and Haber-Weiss reactions consist of reduction of H_2O_2 by transition metal ions, especially ferrous ion (Fe^{2+}) and to a lesser extent, copper (Cu^{2+}) and other ions. Fe^{+2} is oxidized to Fe^{+3} (ferric ion) very easily, and this one is very insoluble. Therefore, free iron that may exist in biological systems will be in very small concentrations and under its ferric form (Halliwell and Gutteridge, 1986). Fenton (Fenton, 1894) has discovered that it is possible to oxidize organic molecules from mixtures of hydrogen peroxide and Fe^{2+} (Fenton's reagent). Thereafter, Haber and Weiss gave an initial explanation of the reaction mechanism: the Fe^{2+} reduces H2O2, which in turn decomposes itself to hydroxyl radical and hydroxyl ion (Haber and Weiss, 1932). In another reaction, Fe^{3+} reacts with $O_2^{\bullet-}$ to produce Fe^{2+} and O_2. This can be represented as the following cycle leading to a continuous $HO^{\bullet}$ production.

Figure 12. Fenton-Haber-Weiss $HO^{\bullet}$ cycle production.



Various studies observed an increased intramuscular free iron concentration associated with an impaired function of enzymes involved in iron metabolism (e.g. heme-oxygenase) in aged skeletal muscle from rodents and humans (Altun et al. 2007; Jung et al. 2008; Xu et al. 2008; Hofer et al. 2008; Safdar, deBeer, et al. 2010). This phenomenon would lead to increase $HO^{\bullet}$ production which would explain in part the increase in muscle oxidative damage to DNA, RNA, lipids and proteins observed in these studies (Jung et al. 2008; Xu et al. 2008; Hofer et al. 2008; Safdar, deBeer, et al. 2010). Finally, Xu et al. (2008) and Hofer et al. (2008) showed in rats that increased intramuscular free iron and increased oxidative damage were associated with decreased gastrocnemius weight and decreased grip strength.

2.1.3. Increased Xanthine oxidase activity as source of RONS

Xanthine oxidase (XO) and xanthine dehydrogenase (XDH) are two isoenzymes of xanthine oxidoreductase (XOR) involved in the catabolism of purines. Indeed, they catalyze the oxidation of hypoxanthine and xanthine to uric acid (a powerful antioxidant). The XOR can generate RONS through different reactions. While during the oxidation process XDH preferentially transfers electrons to NAD^+, XO uses oxygen for this process thus producing $O_2^{\bullet-}$ (Hellsten et al 1988). XO can produce two molecules of $O_2^{\bullet-}$ and one of H_2O_2 for each molecule of NADH oxidized. By the same reaction, the XO can also catalyze the formation of $NO^{\bullet}$ from nitrite. Naturally, XOR is synthesized as XDH and remains mostly as such in the cell, but can quickly become XO by oxidation of sulfhydryl residues and mainly through the activation of calcium-dependent protease (Della Corte and Stirpe, 1968). In healthy tissue, between 10 and 30% of the total activity of the enzyme proceeds as XO (Chambers et al., 1985), but under certain conditions such as aging, it may occurring a conversion of XDH to XO which would lead to increased production RONS. Indeed, regardless muscle type (oxidative or glycolytic), several studies have observed an increase in XO activity in sarcopenic muscle (Lambertucci et al. 2007; Hofer et al. 2008; Ryan et al. 2011). This latter was associated with an increase in muscle content RONS (Ryan et al. 2011) and increased oxidative damage of lipids, proteins and RNA (Lambertucci et al. 2007; Hofer et al. 2008; Ryan et al. 2011). Ultimately these studies have shown an association between increased XO activity, increased oxidative damage and decreased muscle weight (Hofer et al. 2008), maximum aerobic speed (Lambertucci et al. 2007) and also muscle strength (Ryan et al. 2011). This increased XO activity in sarcopenic muscle could be explained by an increase in the activity of calcium-dependent protease responsible for the conversion of XDH to XO. Although to our knowledge no study has measured their activity, some studies have shown an increase in intramuscular concentrations of Ca^{2+} during aging (Fraysse et al. 2006; Andersson et al. 2011) that could increase the activity of these proteases and therefore the conversion of XDH to XO. Andersson et al. (2011) showed in animal sarcopenic muscle an increased RyR1 receptor (Ryanodine receptor 1) oxidation and nitrosilation. These alterations were associated with an increase in cytosol Ca^{2+} release, oxidative damage and a decrease in strength and running capacity (Andersson et al. 2011).

2.1.4. NADPH Oxidase and Nitric oxide Synthase as sources of RONS ?

The NADPH oxidase (NOX) located within the sarcoplasmic reticulum, transverse tubules and sarcolemma is an important source of biological production of free radicals (see figure 11). It is also strongly present in polymorphonuclear neutrophils and macrophages (see figure 11). These latter consummate a lot of oxygen and therefore their activation during an inflammatory condition is causing a production of free radicals. While useful in the inflammatory reaction, they can cause oxidative damage to the surrounding cells. Marzani et al. (2008) showed in old rats a decrease in the weight of hind limbs muscles associated with a chronic systemic inflammatory state. In addition, a recent study has shown in sarcopenic mice (as evidenced by a decreased gastrocnemius weight) an increase of the NOX gastrocnemius protein content associated with an increased $O_2^{\bullet-}$ and H_2O_2 gastrocnemius content (Sullivan-Gunn & Lewandowski 2013). Although having no published data to identify their older rats as sarcopenic, Bejma and Ji (1999) showed in these latter a doubling ROS production *via* NOX.

Nitric Oxide synthase (NOS) present in cytosol also appears as a possible source of RONS during sarcopenia. Indeed, it has been reported an increase of the NOS protein content in the atrophied gastrocnemius of sarcopenic mice (Braga et al. 2008). In addition, many studies reported an increase in oxidative damage caused by RNS (i.e. 3-nitrotyrosine) in the muscle of sarcopenic rodents (Jung et al. 2007; Marzetti, Wohlgemuth, et al. 2008; Murakami et al. 2012; Andersson et al. 2011). Finally, the negative correlation between the amount of 3-nitrotyrosine and the quadriceps weight in the sarcopenic animals reported by Murakami et al. (2012) supports the idea that the production of RNS by NOS is involved in sarcopenia.

2.2. Increased oxidative damage in skeletal muscle is associated with sarcopenia

RONS overproduction in sarcopenic muscle leads to an increase in oxidative damage to cellular components. In Human and animals, increased oxidative damage is negatively correlated with sarcopenia parameters such as muscle mass (Murakami et al. 2012), strength (Howard et al. 2007) and walking speed (Semba et al. 2007).

2.2.1. Protein oxidative damage: Protein carbonylation and nitrosylation

The RONS can attack proteins by damaging their tertiary structure, by fragmenting, by oxidizing the thiol residues (-SH) and altering different amino acids (Davies & Delsignore 1987). Among the various forms of oxidation, carbonylation (adding a carbonyl group, C=O) is one of the most studied and reflects the irreversible oxidation that affects mainly arginine, threonine, proline and lysine. Undergoing significant changes in their conformation, the oxidized proteins generally become more sensitive to the action of proteases and therefore are gradually eliminated (Yu 1994). Several techniques are used to assess the protein carbonylation. Results and information obtained differ depending on the technique used. Results obtained with spectrophotometric techniques (global measure) are contradictory. Indeed, studies in rodents did not highlight differences in the total content or mitochondrial carbonylated protein during sarcopenia (Capel et al. 2004; Mosoni et al. 2004) whereas others showed an increase in protein carbonylation in sarcopenic elderly (Safdar et al. 2010) and mice (Jackson et al. 2011). The Western blotting technique led to improve the analysis by differentiating proteins according to their molecular weights and numerous studies showed an increase in protein carbonylation in sarcopenic elderly (Barreiro et al. 2006) and rodents (Clavel et al. 2006; Muller et al. 2006; Hepple et al. 2008). More recently, development of 2D electrophoresis techniques coupled to mass spectrometry techniques and immune-precipitation have identified specific carbonylated protein during sarcopenia. Indeed, it has been found that mitochondrial proteins are a privileged target of carbonylation (Feng et al. 2008) as well as the ryanodine receptor (RYR1) (Anderssen 2012). It was also observed that a greater number of carbonylated proteins appeared with age in type II fibers because they present lower antioxidant defenses (Feng et al. 2008). Since these proteins are involved during muscle contration, it is not surprising that carbonylated proteins are negatively correlated with strength (Howard et al. 2007) and walking speed (Semba et al. 2007) in sarcopenic elderly.

The 3-nytrotyrosine (3-NT) is another marker of protein damage, which is increasingly used. It reflects protein nitrosilation which is a marker of oxidative damage caused by the RNS. It is formed when the tyrosine is nitrated by peroxynitrite ($ONOO^{\bullet}$). Several studies have shown that during sarcopenia or aging there is an increase in protein nitration in human muscle (Barreiro et al. 2006) and rodent muscles (Jung et al. 2007; Marzetti, Wohlgemuth, et al. 2008; Andersson et al. 2011; Murakami et al. 2012). Some studies have identified *some of these molecules:* creatine kinase (Nuss et al. 2009; no data of sarcopenia parameter), SERCA2 (Fugere et al. 2006; no data of sarcopenia parameter) and RYR1 (Andersson et al. 2011). In

addition, Murakami et al. (2012) showed a negative correlation between 3-NT and muscle mass in sarcopenic rats.

2.2.2. Lipid oxidative damage: Lipid peroxidation

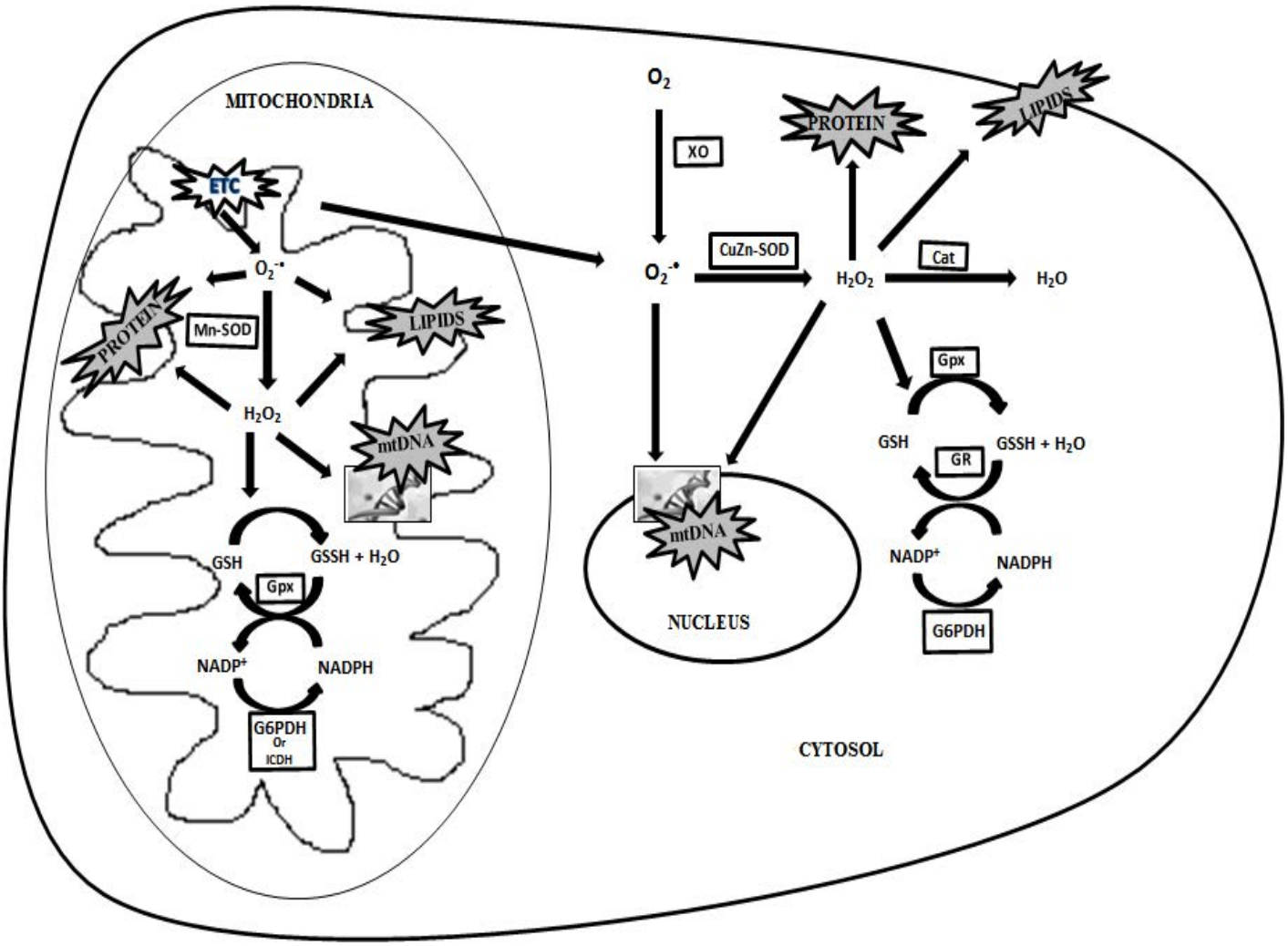
Lipid peroxidation refers to reactions between free radicals and polyunsaturated fatty acids (PUFA) particularly those of the plasmatic membrane, leading to their oxidation. It involves three processes: the initiation phase, propagation and termination. The initiation phase is the creation of a fatty acid radical from a fatty acid, this by the abstraction of a hydrogen atom. Then, the fatty acid radical undergoes molecular rearrangement to give a conjugated diene structure which is more stable. During the propagation phase, the fatty acid radical becomes peroxyl radical ($ROO^{\bullet}$) by addition of oxygen molecule at its centered carbon. This peroxyl radical is sufficiently reactive to remove hydrogen again to a second PUFA which results in the formation of a lipid hydroperoxide (ROOH). The ROOH formed can be rapidly oxidized in the presence of iron or copper, which results to the formation of aldehydes and alkanes. The termination phase stops this chain reaction by the combination of two free radicals which form a more stable compound, or most commonly by reacting with an antioxidant molecule. Its extent can be assessed through the measurement of various markers including thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and its by-products (e.g. 4-hydroxy-2-nonenoic acid, HNA) and isoprostanes (the gold standard). MDA and 4-HHNE protein adducts are also used. Sarcopenia is associated with an increased concentration of lipid peroxidation markers in skeletal muscle in humans (Barreiro et al. 2006; Safdar, deBeer, et al. 2010) and rodents (Muller et al. 2006; Kim et al. 2008; Kovacheva et al. 2010; Ryan et al. 2011). To our knowledge, there is no study which has shown a correlation between lipid peroxidation and sarcopenia.

2.2.3. Nucleic acids oxidative damage

Nuclear and mitochondrial DNA and RNAs are also targets of OS. Among the components of DNA and RNAs, thymine and cytosine are more susceptible to oxidative damage, followed by adenine, guanine and the molecules of deoxyribose (DNA) and ribose (RNA) (Yu 1994). Oxidative damage to nucleic acids may result in cellular dysfunction as well as transcriptional and translational anomalies multiplication. The main technique used to measure the oxidation of nucleic is based on the determination of the compounds formed by the hydroxylation of bases: 8-oxo-deoxyguanosine (8-OHdG) for DNA and 8-oxo-

oxyguanosine (8 -OHG) for RNA. Aging and sarcopenia are associated with increased level of oxidative damage to DNA in skeletal rodents and human muscle. This damage appears to particularly affect mtDNA, probably because of the large RONS mitochondrial overproduction in aged muscle tissue (as previously described). Indeed, it has been reported that level of oxidized bases was 2-3 times higher at the mtDNA than nuclear DNA, despite a higher repair capacity of DNA in mitochondria (Stevnsner et al. 2002). This is one of the major factors involved in mitochondrial dysfunction developing with age. Many studies reported an increase in muscle 8-OHdG content in rodents during sarcopenia (Mansouri et al. 2006; Muller et al. 2006; Ryan et al. 2008; Xu et al. 2008). In addition, it has also been reported in rodents an increased 8-OHG muscle content (Xu et al. 2008). In humans, there is an increased 8-OHdG content in aged muscle (Mecocci et al 1999 Fano et al 2001) but to our knowledge no study has measured this parameter and indicators of sarcopenia (e.g. muscle mass, strength) in the same work.

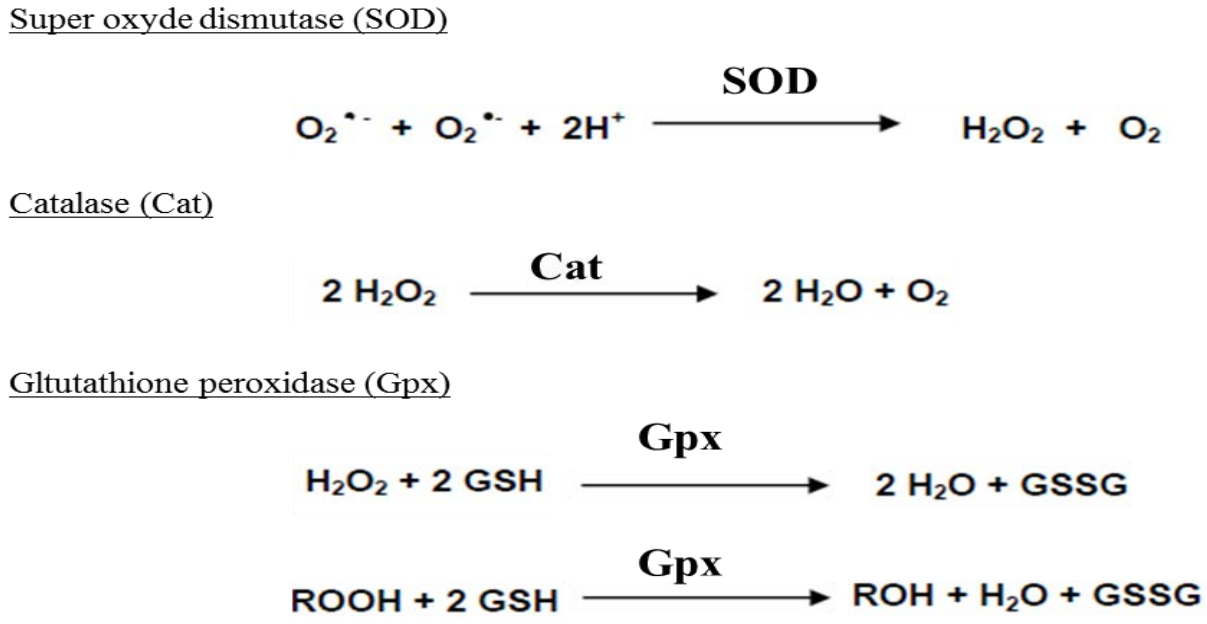
Figure 13. Schematic representation of RONS source, antioxidant systems and oxidative damage.



2.3. Antioxidant defenses, aging and sarcopenia

The organism has several antioxidant defenses systems designed to protect against RONS' action. Antioxidants can be classified in several ways. From a cell physiology point of view, they can be divided into primary, secondary and tertiary antioxidants. The first prevent the formation of new free radicals by converting existing free radicals into less harmful molecules or preventing its formation through others molecules. Among them (figure 13 and 14), there are at least SODs, glutathione peroxidase (Gpx), glutathione reductase (GR), γ-glutamate-cysteine ligase (γ-GCLC), glucose-6-phosphate dehydrogenase (G6PDH), catalase (Cat) and metal binding proteins such as heme oxygenase. The second ones are non-enzymatic protector or free radical scavengers which act when there is an overproduction of free radicals and when the enzymatic systems are overwhelmed, preventing chain reactions. They include at least glutathione, vitamin E (i.e. alpha-tocopherol), vitamin C, carotenes (vitamin A), uric acid, bilirubin, and albumin. Finally, the last ones repair biomolecules damaged by free radicals. They include intracellular proteolytic systems which act to degrade oxidatively damaged proteins thereby preventing their accumulation (Davies, 1987; Pacifi and Davies, 1991), DNA-repair enzymes (e.g. oxoguanine DNA glycosylase), protein-repair enzymes (e.g. thioredoxin) and lipid-repair enzymes (e.g. phospholipase A2). From a biochemical point of view, antioxidant systems are classified as enzymatic antioxidants, non-enzymatic antioxidants and repair systems.

Figure 14. Reactions of the main antioxidant enzymes.



2.3.1. Enzymatic antioxidant systems are impaired during aging and sarcopenia

According to studies cited in table 10, it appears that during aging and sarcopenia, regulation of antioxidant enzymes would not happen to transcriptional level. Indeed, studies generally show no change in RNA coding for antioxidant enzymes (Ryan et al. 2008; Ryan et al. 2011). Data on protein content of antioxidant enzymes are disparate. In general, whatever the concerned specie (e.g. humans or rodents, protein content of enzymes which directly convert free radicals in less reactive molecules (e.g. CuZn-Sod, Mn-Sod and Cat) does not vary (Ryan et al. 2008; Kim et al. 2008; Jackson et al. 2010; Ryan et al. 2011; Jackson et al. 2011) while protein content of enzymes involved more indirectly in RONS elimination as G6PDH or γ-GCLC decreases (Kumaran et al. 2004; Braga et al. 2008; Kumaran et al. 2008; Kovacheva et al. 2010; Safdar et al. 2010). At the mitochondrial level, the activity of Mn-SOD and GPx is increased during aging and sarcopenia (Ji et al. 1990 ; Marzani et al. 2005; Ryan et al. 2011). Acting in synergy, these adaptations could be a defense mechanism to support mitochondrial overproduction of $O_2^{-\bullet}$ and H_2O_2 described previously. Concerning the cytosolic activities of CuZn-SOD, Cat and Gpx, the results seem depend on the species studied. Indeed, studies mostly report a higher activity of CuZn-SOD, Cat and Gpx in muscles of aged rodents (Ji et al. 1990; Ryan et al. 2008; Jackson et al. 2010; Ryan et al. 2011), whereas studies in humans generally observed no change (Pansarasa et al. 1999; Gianni et al. 2004; Marzani et al. 2005). Longitudinal studies examining the activity of antioxidant enzymes in the muscle are few but can distinguish several phases in life. Generally in rats it appears that the activity of antioxidant enzymes decreases from 3-6 months (reaching adulthood) to 18-21 months (onset of sarcopenia), increases after 22-24 months until very advanced ages (Ji et al. 1990; Lawler & Demaree 2001; Mosoni et al. 2004; Sullivan-Gunn & Lewandowski 2013).

Table 10. Sarcopenia-associated enzymatic antioxidant defenses impairment in skeletal muscle.

Reference	Specy	Age	Compartment	Enzyme
Barakat et al. 1989	Rats	3 m *vs* 18 m	Cytosolic fraction	G6PDH activity ↘
Ji et al. 1990	Rats	4 m *vs* 31 m	Cytosolic Fraction Mitochondrial fraction	G6PDH, GR, Gpx, CuZn-SOD, Cat activity ↗ Gpx and Mn-SOD activity ↗
Pansarasa et al. 1999	Human	17-25 y *vs* 66-75 y 17-25 y *vs* > 76 y	Whole muscle homogenate Mitochondrial fraction Cytosolic fraction	Total SOD activity ↘ Mn-SOD activity ↗ Gpx and Cat activity no change
Gianni et al. 2004	Human	22 y *vs* 72 y	Whole muscle homogenate	Mn-SOD activity ↗ Cat and CuZn-SOD activity no change
Marzani et al. 2005	Human	18-48 y *vs* 66-90 y	Mitochondria fraction Cytosolic fraction	Mn-SOD activity ↗ CuZn-SOD, Gpx and Cat activity no change
Kumaran et al. 2004	Rats	3-4 m *vs* 24 m	Whole muscle homogenate	Gpx, GR and G6PDH activity ↘
Barreiro et al. 2006	Human	25 y *vs* 68 y	Total muscle homogenate	Mn-SOD and Cat content ↗
Ryan et al. 2008	Rats	3 m *vs* 30 m	Cytosolic fraction Whole muscle homogenate	Mn-SOD, CuZn-SOD, Cat and Gpx content no change Mn-SOD, CuZn-SOD, Cat and Gpx RNA no change Mn-SOD, CuZn-SOD and Gpx activity no change Cat activity ↗
Kumaran et al. 2008	Rats	3-4 m *vs* 24 m	Whole muscle homogenate	Total SOD, Cat, Gpx, GR and G6PDH activity ↘
Kim et al. 2008	Rats	6 m *vs* 24 m	Whole muscle homogenate	Mn-SOD content no change CuZn-SOD content ↗
Braga et al. 2008	Mice	5 m *vs* 25 m	Whole muscle homogenate	G6PDH content ↘
Kovacheva et al. 2010	Rats	2 m *vs* 22 m	Whole muscel homogenate	G6PDH content ↘
Safdar et al. 2010	Human	22 y *vs* > 63 y	Whole muscle homogenate	Heme oxygenase and γ-GCLC content ↘
Jackson et al. 2010	Rats	6 m *vs* 34 m	Whole muscle homogenate	Mn-SOD, CuZn-SOD and Cat content no change Mn-SOD, CuZn-SOD and Cat activity ↗
Ryan et al. 2011	Mice	3-5 m *vs* 26-28 m	Mitochondrial fraction Whole muscle homogenate Free mitochondrial fraction	Mn-SOD activity ↗ Mn-SOD content no change Gpx, Mn-SOD and CuZn-SOD RNA no change Cat RNA ↗ Gpx activity no change Cat activity ↗ CuZn-SOD content no change ↗ Cat content
Jackson et al. 2011	Mice	3 m *vs* 28 m	Mitochondrial fraction Cytosolic fraction Whole muscle homogenate	Mn-SOD activity no change CuZn-SOD activity ↗ CuZn-SOD content ↗

G6PDH: Glucose-6-Phosphate Dehydrogenase; GR: Glutathione Reductase; Gpx: Glutathione Peroxidase; CuZn-SOD: Copper-Zinc Super oxide dismutase (mostly present in cytosol); Cat: Catalase; Mn-SOD: Manganese Super oxide dismutase (mostly present in mitochondria); γ-GCLC: γ-Glutamyl cysteine synthase.

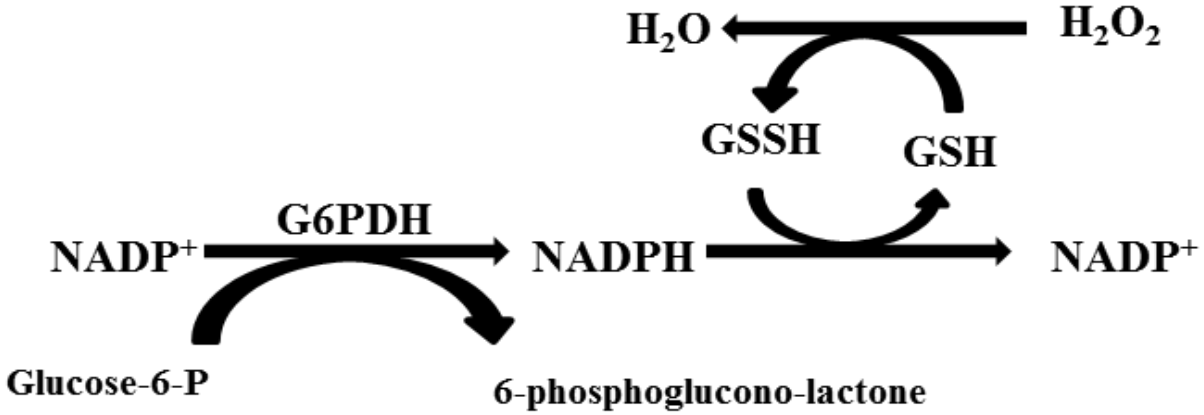
Finally, sarcopenia seems to appear during a life period when antioxidant defenses are weakened and RONS production increased which would lead to oxidative damage as previously described. Thereafter, these defenses increase but the persistence of oxidative damage shows that this increase is not enough to counteract the overproduction of RONS.

2.3.2. Non enzymatic antioxidant systems are impaired during aging and sarcopenia

Glutathione is the most abundant non-protein thiol in muscle cells. Its active group is the sulfhydryl of the cysteine residue by which glutathione may exert a protective role when present in its reduced form (GSH). It contributes to the reduction of H_2O_2 in water via a system involving GPx, GR and G6PDH, but can also scavenge spontaneously some radical species (see figure 13 and 15). Two molecules of GSH can be oxidized giving an electron each other. Then they fuse between them to form a disulfide form (GSSG). Thereby, a characteristic indicator of oxidative stress is the increased concentration of oxidized glutathione, with the consequent alteration of the redox state of glutathione, increasing the GSSG/GSH (Sies 1986). Data on total glutathione, GSH and GSSG muscle content are contradictory (Pansarasa et al. 1999; Mosoni et al. 2004; Marzani et al. 2005). In contrast, studies agree on an increase in GSSG/GSH (Kumaran et al. 2004; Marzani et al. 2005; Kumaran et al. 2008; Ryan et al. 2008; Ryan et al. 2011). Moreover, studies have reported a decrease in the activity and/or protein content of some molecule involved in the synthesis and/or regeneration system of GSH during sarcopenia as G6PDH, GR (Kumaran et al. 2004; Mosoni et al. 2004; Braga et al. 2008; Kumaran et al. 2008; Kovacheva et al. 2010; Safdar et al. 2010). Taken together, these data suggest that sarcopenia is associated with an impaired glutathione system.

Some vitamins are part of the non-enzymatic antioxidant systems but there is lack of data concerning their involvement in sarcopenia. Nevertheless, it seems that a deficient status in vitamins E (i.e. alpha-tocopherol) and carotenes would be a factor favoring the onset of sarcopenia (Semba et al. 2003).

Figure 15. Gluthatione system representation.



2.3.3. Repair systems seem to be impaired during aging

Some oxidative damage are sometimes reversible and can be supported by repair systems. For example, enzyme such as thioredoxin (Trx) is able to repair oxidative damage in some protein level as the oxidation of cysteine (Ugarte et al. 2010). However, aging is associated with reduced muscle expression of this enzyme thus suggesting that the operation of this repair system is altered (Rohrbach et al. 2006). Oxidative damage to DNA can also be supported by some enzymes as oxoguanine DNA glycosylase (OGG1) (Bohr et al. 2002). Although few data on the subject are available, it appears that aging is also responsible for a reduction in the activity of OGG1 in skeletal muscle (Koltai et al. 2010). Those results would be extrapolated to sarcopenia because they were obtained with senescent animal in which sarcopenia is usually described. In other cases, oxidative damage is irreversible and damaged cell components must be removed to avoid further cell damage. In the case of proteins, proteolytic and autophagic systems (as previously described) will ensure this degradation. These systems are optimized with the heat shock proteins (HSPs). These stress protein expressed in all cellular compartments work as chaperone molecules. They facilitate protein folding avoiding protein aggregation. Data on the effect of aging on their muscle protein content are controversial. Indeed, studies observed an increase (Siu et al. 2006; Thalacker-Mercer et al. 2010) while other showed any modification (Vasilaki et al. 2006; Gupte et al. 2008). More data are needed to make a conclusion on this subject.

2.4. Mechanistic links between oxidative stress and sarcopenia

OS may contribute to activating or inhibiting molecular signaling pathways involved in sarcopenia supporting the cell signaling disruption theory of aging exposed by (Viña et al. 2013). Moreover, OS might alter the contractile qualities of muscle, regardless of muscle atrophy (Reid 2008).

2.4.1. Link between oxidative stress and impaired satellite cells activity

Impaired satellite cells activity would contribute to sarcopenia by limiting the incorporation of new nuclei in muscle fiber to replace the damaged nuclei.

Numerous studies consider that the cellular environment of the old muscle is responsible for alterations in the activity of SC more than the intrinsic myogenic potential of these latter (Carlson & Faulkner 1989; Carlson, Suetta, et al. 2009). Thereby, recent studies demonstrated in C2C12 cells that reducing the redox environment promotes both proliferation

(Renault et al. 2002) and myoblast differentiation (Ardite et al. 2004; Hansen et al. 2007) underlying the importance of RONS in these processes.

On the other hand, studies have also suggested that the decreased activity of SC in aged muscle may be related to increased oxidative stress within SC (Fulle et al. 2005; Beccafico et al. 2007). Indeed, Fulle et al. (2005) showed that antioxidant enzymes activity is decreased in satellite cells extracted from old men (more than 70 years old) compared to young men (30-40 years old). The lipids peroxidation higher in old myotubes obtained from old SC was associated with a decreased myoblast fusion capacity to generate myotubes (Beccafico et al. 2007).

2.4.2. Oxidative stress could disturb protein turn-over

Theoretically, oxidative stress can contribute to disuse muscle atrophy by depressing protein synthesis and/or increasing proteolysis.

In regard to RONS and decreased protein synthesis, some studies have shown impairment of the PI3K/Akt/mTOR pathway associated with an increase of OS. For instance, Clavel et al. (2006) and Kovacheva et al. (2010) have shown in old rat that decreased IGF-1 RNA and Akt activation were associated with increased lipids peroxidation and proteins carbonylation in skeletal muscle. Similar data were published in humans by Safdar et al. (2010). On the other hand, decreased oxidative damage were associated with increased Akt activation (Kovacheva et al. 2010). In the same way, a clear increase in postprandial protein synthesis is observed in older rodents treated with antioxidants (Marzani et al. 2008). Emerging evidence suggests that ROS can depress protein synthesis by obstructing mRNA translation at the level of initiation (Shenton et al. 2006; O'Loghlen et al. 2006; Zhang et al. 2009). For instance, RONS such as H_2O_2 (known to increase during sarcopenia) appears to impair mTOR assembly and therefore preventing mTOR-mediated phosphorylation of 4E-BP1 and p70S6K in muscle cultured cells (Zhang et al. 2009). Moreover, oxidative DNA damage are known to activate p53 which is able to inhibit mTOR *via* AMPK and TSC2 (Feng et al. 2005).

In regard to RONS and increased proteolysis, growing evidence indicates that oxidative stress can promote muscle protein breakdown by different ways.

First, altered redox status was associated with an increased gene expression of UPS up-stream such as TNF-α (Clavel et al. 2006), UPS effectors such as MuRF1 and Atrogin-1, and proteasome activity (Clavel et al. 2006; Hepple et al. 2008) which was negatively correlated with muscle mass (Hepple et al. 2008). Reports indicate that OS promotes

increased gene expression of key proteins involved in the proteasome system of proteolysis. For example, *in vitro* experiments have demonstrated that exposure of C2C12 myotubes to H_2O_2 (known to increase during sarcopenia) up-regulated the expression of MuRF1 and Atrogin-1(Y.-P. Li et al. 2003). Similarly, TNF-α induced-increase ROS production within myotubes is also associated with increased expression of Atrogin1 and MuRF1 through a p38 MAPK signaling pathway (Li et al. 2005).

Secondly, aging is associated with increased calpains activity in skeletal muscle (Dargelos et al. 2007; Samengo et al. 2012) which could be explained by the sarcopenia-associated increase H_2O_2 production. Indeed, recent studies revealed that oxidative stress through H_2O_2 can increase the expression and activity of calpains 1 and 2 in both C2C12 myotubes and human myoblasts (McClung et al. 2009; Dargelos et al. 2010). On the other hand, aging is associated with a skeletal muscle cytosolic calcium overload (Fraysse et al. 2006) known to increase calpains activity (Goll et al. 2003). ROS production could play an important role in disturbances in calcium homeostasis (Kandarian & Stevenson 2002). A potential mechanism to link oxidative stress with calcium overload is that ROS-mediated formation of reactive aldehydes (i.e. 4-hydroxy-2,3-trans-nonenal) can inhibit plasma membrane Ca^{+2} ATPase activity which would lead to intracellular Ca^{+2} accumulation (Siems et al. 2003). In another way, increased lipids peroxidation in old rodents skeletal muscle is associated with an increased caspase-3 and muscle atrophy (Wohlgemuth et al. 2010). Recent reports indicate that oxidative stress can activate caspase 3 in muscle fibers *in vitro* and *in vivo*. For example, exposing C2C12 myotubes to H_2O_2 (known to increase durin aging) has been shown to activate caspase 3 (Siu et al. 2009). Notably, new evidence reveals that antioxidant-mediated protection against inactivity-induced oxidative stress prevents caspase-3 activation in diaphragm muscle *in vivo* (Whidden et al. 2010).

Concerning OS and autophagy data are contradictory. Evidence suggests that increased cellular ROS production and over increased SOD activity in skeletal muscle of transgenic mice promotes the expression of autophagy-related genes (e.g. Beclin-1 and cathepsin L) (Thorpe et al. 2004; Dobrowolny et al. 2008). However, in atrophied muscle of old rat with high level of lipid peroxidation, while several autophagy related proteins were up-regulated (Beclin-1), others were down-regulated (LC3) (Wohlgemuth et al. 2010). Moreover, as exposed in the chapter 2, almost all studies seem to agree on a reduction in protein degradation *via* autophagy in muscle aging and sarcopenia in humans and animals (McMullen et al. 2009; Wohlgemuth et al. 2010; O'Leary et al. 2013; Fry et al. 2013; Kim et al. 2013). More data appear necessary to establish the relation between OS and autophagy.

Finally, ROS can also accelerate proteolysis in muscle fibers by oxidizing muscle proteins, which enhances their susceptibility to proteolytic processing. Indeed, using several purified proteases, Davies (1987) first demonstrated that ROS accelerate the protease-mediated breakdown of proteins. This observation has been expanded by others, and it is now established that oxidized proteins are readily degraded by many proteases, including the 20S proteasome, calpains, and caspase 3 (Grune et al. 2003; Smuder et al. 2010). In particular, oxidation increases myofibrillar protein breakdown in a dose-dependent manner and following oxidative modification, MHC, α-actinin, actin, and troponin I are all rapidly degraded by calpains (I and II) and caspase-3 (Smuder et al. 2010).

2.4.3. Oxidative stress and muscle contractile qualities

The decrease of strength in the aged-muscle is not only explained by muscle atrophy but also by alterations in contractile properties.

RONS are recognized as involved in the regulation of muscle strength (Reid 2008). Low basal RONS concentrations are necessary for muscle contraction and an optimum RONS concentration is necessary to reach the maximum of muscle force (Reid 2001). However, muscle contraction is altered when RONS concentrations are too high (Reid 2001).

With regards to these results, it is not surprising that different studies observed a decreased maximal isometric strength and an increased fatigability in skeletal muscle of old rats associated with a concomitant increased RONS production (Chabi et al. 2008; Jang et al. 2010; Andersson et al. 2011). The cellular and molecular target leading to the muscle strength deterioration in case of RONS overproduction are still poorly known. However, several proteins involved in the excitation contraction coupling have been shown to be more carbonylated and/or nitrozylated such as SERCA 2 (Fugere et al. 2006) and RyR1(Andersson et al. 2011).

It has been suggested that high RONS concentrations could affect the release of intracellular calcium or calcium sensitivity of contractile myofilaments (Smith and Reid 2006; Zima and Blatter 2006). Indeed, in old rats, Andersson et al. (2011) showed that EDL specific strength was associated with a RyR1 increased carbonylation and nitrozylation, surely due to the concomitant increased RONS content. Then, RyR1 oxidative modifications were associated with an increased intra-cellular Ca^{2+}.

More studies are needed to highlight the mechanisms by which RONS production altered muscle contractile quality during sarcopenia.

3. Chapter 3 abstract

Both in humans and animals, it has been showed that sarcopenic muscle exhibits increased RONS production (e.g. $O_2^{\bullet-}$ et H_2O_2) (Capel et al. 2004; Capel, Rimbert, et al. 2005; Capel, Demaison, et al. 2005; Chabi et al. 2008; Jackson et al. 2011; Andersson et al. 2011; Miller et al. 2012) and content (Andersson et al. 2011; Janna R. Jackson et al. 2010; Jackson et al. 2011; Ryan et al. 2011; Sullivan-Gunn & Lewandowski 2013). This overproduction of RONS is mainly due to mitochondrial dysfunctions (Capel, Rimbert, et al. 2005; Chabi et al. 2008) and increased xanthine oxidase activity (Lambertucci et al. 2007; Ryan et al. 2011). Although NADPH oxidase and nitric oxide synthase muscle protein content is increased in sarcopenic muscle, increased RONS production by these latter have to be confirmed (Sullivan-Gunn & Lewandowski 2013; Braga et al. 2008). RONS overproduction in sarcopenic muscle leads to an increase in oxidative damage to cellular components (lipid plasma membranes, proteins and nucleic acids). In both humans and animals, increased oxidative damage is negatively correlated with sarcopenia parameters such as muscle mass (Murakami et al. 2012), strength (Howard et al. 2007), walking speed (Semba et al. 2007).

Increased oxidative damage reflect the inability of antioxidant systems to contain overproduction of RONS and attest an imbalance of the "oxidants-antioxidants" balance leading to an impaired redox homeostasis, known as oxidative stress (Sies 1985; Jones 2006).This impaired redox status may be the cause of the disturbance of a number of intracellular signaling pathways involved in sarcopenia. *In vitro* studies showed that the oxidative stress would disturb protein synthesis and stimulates several cellular mechanisms involved in muscle atrophy as proteolysis or alteration of muscle regeneration. Chronic oxidative stress observed in aged muscle could promote these mechanisms and lead to sarcopenia. Nevertheless, the link between these cellular mechanisms involved in sarcopenia and oxidative stress needs to be clearly demonstrated *in vivo* in the old muscle tissue.

As it will presented in the following chapter, effective strategies to fight against sarcopenia such as exercise would restore a “young” redox status.

Chapter 4: Strategies against sarcopenia

Developing strategies for the prevention and treatment of sarcopenia will not only help to enhance the quality of life for individual patients who suffer from this syndrome but also for reduction in economic and productivity burdens would be beneficial to society as a whole. As exposed in the three first chapters, sarcopenia is characterized by a decreased muscle mass, strength and physical performance. Impaired protein turnover, mitochondrial dysfunctions, exacerbation of apoptosis and impaired satellite cells functions are mechanisms which can explain in part the onset and development of this syndrome. Neuromuscular dysfunctions are also involved (Edström et al. 2007) but are beyond the scope of this work. Oxidative stress appears to be involved in these mechanisms as well as a decrease in the production of anabolic hormones (GH, IGF-1, and testosterone).

The identification of cost-effectiveness interventions to maintain muscle mass and physical functions in the elderly is one of the most important public health challenges. In this chapter, we will present the available evidence regarding the impact of physical exercise and alternative strategies such as antioxidant and hormone replacement strategies on the components of sarcopenia. For each strategy, we will present data about the mechanisms by which it act on sarcopenia.

1. Exercise as the perfect strategy against sarcopenia

Exercise appears to be the perfect strategy against sarcopenia because it can lead to an in increase muscle mass, strength and physical performance (Pillard et al. 2011; Di Luigi et al. 2012; Wang & Bai 2012; Montero & Serra 2013). In this work, when not specified exercise will refer to a repetition of different exercise sessions (i.e. training). Exercise have also positive effects on the metabolic, cardiovascular and reproductive systems (Pillard et al. 2011; Di Luigi et al. 2012; Wang & Bai 2012; Montero & Serra 2013). In addition, exercise is known to improve quality of life, psychological health and is associated with better mental health and social integration, improves anxiety, depression and self-efficacy in older adults (Mather et al. 2002). Usually, four type of exercise are recommended for older adults to prevent sarcopenia: aerobic (endurance), resistance (strength), flexibility (stretching) and balance (proprioception) training. Recommendations about the prescription of exercise to the elderly is not the objective of this part, however, we recommend the following papers for

review: Pillard et al. 2011; Di Luigi et al. 2012; Wang & Bai 2012; Montero & Serra 2013. Here, we will focus on the mechanisms by which exercise (essentially resistance and endurance exercise) lead to combat against sarcopenia.

1.1. Exercise during aging improves protein turnover

Although whole body protein synthesis appeared to be unchanged by resistance exercise in older people (Yarasheski et al. 1993; Welle et al. 1995; Hasten et al. 2000), numerous studies found that this exercise can increase specifically mixed muscle protein synthesis (Welle et al. 1995; Balagopal & Schimke 2001; Yarasheski et al. 1993; Yarasheski et al. 1999; Hasten et al. 2000; Short et al. 2004) in particular myofibrillar proteins (Welle et al. 1999) such as MHC (Welle et al. 1995; Hasten et al. 2000; Balagopal & Schimke 2001). In response to resistance exercise, these increases are always associated with improvement of muscle mass and strength increase (Welle et al. 1995; Welle et al. 1999; Balagopal & Schimke 2001; Yarasheski et al. 1993; Yarasheski et al. 1999; Hasten et al. 2000). Typically, resistance training programs used in the cited studies lasted 3-4 months, with 3 sessions per week (separated by a rest day) with 2-3 sets of multiple exercises alternating between high and low body, at gradually increasing intensities from 50-60% to 75-80% of 1RM. However, one week of resistance exercise is sufficient to obtain these results and with only two weeks, the beneficial effect will persist even for 3 months (Hasten et al. 2000). Interestingly, although resistance training is typically associated with the most profound gains in strength, elderly subjects who completed a 3 months moderate intensity aerobic program (3-5 days per week, with sessions of 20-45 minutes at 60-80% of the heart rate reserve) also demonstrated marked increases in whole muscle size and strength associated with increased mixed muscle protein and MHC synthesis (Short et al. 2004; Konopka et al. 2011). Although there are many studies that have shown an increase in muscle protein synthesis after resistance and aerobic training in the elderly, few studies have investigated the signaling pathways involved in this phenomenon. It seems nevertheless that an activation of the PI3K/Akt/mTOR pathway is involved. Indeed, Mayhew et al. (2009) and Williamson et al. (2010) showed in elderly people that resistance exercise (12-16 weeks, 3 days per week, 80% of 1RM) lead to muscle hypertrophy (CSA increase), increase muscle strength and a substantial muscle protein accretion associated with an increased Akt, p70S6K and rpS6 phosphorylation. Data obtained in hypertrophied skeletal muscle of old rats indicated similar mechanisms. Indeed, chronic muscle overload induced by bilateral ablation of the gastrocnemius for 28 days increased plantaris weight in aged animals associated with an increase in mTOR and rpS6

phosphorylation (Chalé-Rush et al. 2009). On the other hand, it has been shown that aerobic exercise (lifelong running wheel exercise or treadmill training) in old rats increased IGF-1 and IRS-1 protein content in skeletal muscle, and Akt and mTOR activation associated with hypertrophied muscles (Kim et al. 2008; Pasini et al. 2012). Finally, there are very few studies in older people and old animals that have explored the protein synthesis signaling pathways in response to training. More studies are needed to test various types and combinations of training, explore the responses in functions of muscle type (slow or fast) and these evolutions of these responses over the decades.

Until now, various studies have shown that exercise (resistance and aerobic) in elderly subjects has no effect on proteolysis (Yarasheski et al. 1993; Welle et al. 1995; Hasten et al. 2000). This could be explained by different reasons: lack of sensitivity of the used techniques to measure proteolysis; amino acid from proteolysis would be recycled during protein synthesis; exercise increases the activity of several proteolysis systems while other will be decreased at the same time.

To our knowledge, no study has investigated the impact of exercise on calpain system in the elderly or older animals. However, aerobic exercise (life-long voluntary exercise with running wheel) and resistance exercise in old rats (9 weeks, 3 days per week, climbing of a one meter ladder inclined at 85° with weight attached to the tail) lead to decrease caspase 3 activity (Wohlgemuth et al. 2010; Luo et al. 2013). Unfortunately, usual cleaved proteins by caspases such as actin were not measured in these studies.

Data concerning the effect of exercise on the UPS systems are very few but would be consistent with a decrease in the activity of the latter. Indeed, Williamson et al. (2010) showed that resistance training (12 weeks, 3 days per week, 70-75% 1RM) in older people was associated with the nuclear accumulation of FoxO3, but no differences in MuRF1 or MAFbx expression were observed. In the same way, elderly subjects who completed a 12-weeks moderate intensity aerobic program (3-5 days per week, 20-45min per session, 60-80% heart rate reserve) also demonstrated marked increases in whole muscle size and strength associated with a reduction in myostatin and FoxO3 expression, however MAFbx and MuRF1 expression were not different (Konopka et al. 2010). In another study, it has been shown that 4 weeks of supervised endurance training in chronic heart failure patient (mean age 72 years olds) with muscle atrophy, is associated with a decreased of ubiquitinated protein muscle content surely due to the marked decreased in MuRF1 RNA and protein muscle content. As the previous mentioned studies, MAFbx was not affected by exercise. In the same way, LeBrasseur et al. (2009) showed in old mice subjected to a short and low intense treadmill

training (4 weeks, 5 days per week, 20 min per session at 10m/min) a marked decreased in MuRF1 muscle protein content. Finally, all these studies highlighted that exercise in particular aerobic exercise is able to decrease UPS systems compounds associated with a hypertrophic response. However, proteasome activity was never measured in these studies and it can just be hypothesized that exercise would decrease its activity.

The most marked effect on proteolysis of exercise in the elderly and older animals concerns the autophagy regulation. As previously described, sarcopenia is associated with an impaired autophagy, however exercise (endurance as well as resistance training) should reverse this impairment associated with muscle hypertrophy (independently of muscle type) and decreased muscle fatigue (Wohlgemuth et al. 2010; Luo et al. 2013; Kim et al. 2013). Indeed, in response to 8 weeks of treadmill training (5 days per week, 40 min per session at 16,4 m/min), it has been shown an increase of Beclin-1, LC3 and Lamp-2 muscle protein content in old mice associated with an increase in EDL and gastrocnemius weight (Kim et al. 2013). Previously, Wohlgemuth et al. (2010) showed that long life exercised rats presented an up-regulation of Lamp-2 RNA, Atg7 and Atg9 protein associated with an increased plantaris weight. On the other hand, similar result were obtained in response to a resistance training protocol (climbing of a one meter ladder inclined at 85° with weight attached to the tail) in old rats (Luo et al. 2013). Moreover, these autors showed an increased in lysosome protease protein content (i.e. Cathepsin L). Finally, the increase of these different markers suggested that aerobic exercise as well as resitance exercise during aging should stimulate autophagy induction, autophagosome formation and fusion with lysosomes. Indeed, as no studies have directly measured the number of autophagy vesicles, the increase of the different molecules regulating autophagy only suggest an increase of the latter. As autophagy is associated with accumulation of dysfunctional mitochondria and unfolded proteins (previously exposed in the chapter 2), Kim et al. (2013) speculated that exercise training-induced autophagic response might be considered as one of the mechanisms of cellular “clearance” that may be related to protecting against the accumulation of dysfunctional mitochondria and unfolded proteins.

1.2. Exercise during aging decreases apoptosis

Several studies showed that aerobic exercise and resistance training during aging decreased apoptosis associated with muscle mass and strength improvement in old animals (W. Song et al. 2005; Marzetti, Groban, et al. 2008; Wohlgemuth et al. 2010; Luo et al. 2013). However, it seems that no data are available in older humans.

With regard to the effects of exercise training on myonuclear apoptotic signaling, Song et al. (2005) showed that 12-week treadmill exercise (5 days per week, 60 min per session) reduced the expression of Bax in the gastrocnemius muscle of old rats. Conversely, levels of Bcl-2 were increased in exercised rodents, resulting in a dramatic decrease in the Bax-to-Bcl-2 ratio reaching young values. In addition, cleavage of caspase-3 was lowered by 95% in old exercised rats. As a consequence, the extent of gastrocnemius apoptotic DNA fragmentation was significantly attenuated by the exercise intervention, such that old trained rats displayed levels of apoptosis similar to those observed in young control animals. It is noteworthy that the reduced severity of apoptosis was accompanied by an increased fiber CSA associated with an increased muscle weight (soleus and gastrocnemius). Similarly, Marzetti et al. (2008) found that 4-week treadmill exercise training down-regulated the death receptor pathway of apoptosis in the EDL of old rats. Indeed, exercise reversed the age-related increase of TNF-R1, activated caspase-8 and cleaved caspase-3, resulting in reduced levels of apoptotic DNA fragmentation. These adaptations were accompanied by improvements in exercise tolerance and forelimb grip strength. Furthermore, similar data were published by the same group in long life exercised rats with free access to a running wheel (Wohlgemuth et al. 2010). In addition, they showed that exercise reverses the age-related increase of caspase-9 activity. Recently, Luo et al. (2013) found that 9 weeks of resistance training prevented the loss of muscle mass and improved muscle strength, accompanied by reduced cytosolic cytochrome c concentration and inhibited cleaved caspase 3 production resulting in reduced levels of apoptotic index.

Decreased apoptotic myonuclei or DNA fragmentation could be explained by renewal of these latter thanks satellite cells activation.

1.3. Exercise during aging stimulates satellite cells

This topic has been well reviewed by Snijders et al. (2009). Although some studies failed to demonstrate any effect of exercise in older people on satellite cells (Petrella et al. 2006; Leiter et al. 2011) most of them showed an exercise-related activation of these latter in elderly people and older rodents associated with muscle mass and strength improvement (Mackey et al. 2007; Verney et al. 2008; Verdijk et al. 2009; Shefer et al. 2010; Leenders et al. 2013). For instance, Verdijk et al. (2009) found that 3 months of resistance training (3 days per week, 80% 1RM) augmented muscle mass, reduced fat mass, and increases muscle strength in healthy, elderly men. The observed skeletal muscle hypertrophy was specific for the type II muscle fibers and accompanied by a specific increase in Type II muscle fiber

satellite cells content. These data were recently confirmed by Leenders et al. (2013) who showed that 6 months resistance-type exercise training (3 days per week, 80% 1RM) lead to leg lean mass and quadriceps CSA increased resulting in an increased one-repetition maximum leg extension strength and a decreased sit-to-stand time. These results were concomitant to a type II muscle fiber specific increase in myonuclear and satellite cells. On the other hand, Verney et al. (2008) and recently Shefer et al. (2010) found similar results in response to endurance training in elderly (13 weeks of combined lower body endurance and upper body resistance training) and old rats (14 weeks of treadmill training, 6 days per week, 20 min per session) with an increased type II muscle fiber size accompanied by an increase in type II muscle fiber SC content. There is still a debate to know if exercise can directly active satellite cells or if these latter are activated in response to the muscle damage induced by exercise. Studies are needed to bring a conclusion to this debate.

1.4. Exercise during aging improves mitochondrial functions and dynamics

Aerobic exercise of sufficient intensity (at least 60% $\dot{V}O_{2\,max}$) and duration (at least 3 weeks with 3 sessions of one hour per week) can significantly increase $\dot{V}O_{2\,max}$ and endurance capacity in older adults and rodents (Hammeren et al. 1992; Radák et al. 2002; Malbut et al. 2002; Short et al. 2004; Huang et al. 2005; Lambertucci et al. 2007; Lanza et al. 2008; Safdar, Hamadeh, et al. 2010; Koltai et al. 2012). Increases in mitochondrial functions and number, in the expression of mitochondrial proteins and/or in the expression of transcription factors involved in mitochondrial biogenesis are mechanisms whose explain these improvements. Short et al. (2003) were among the first to show in humans that endurance exercise (16 weeks, four sessions per week at 80% of maximal heart rate for 40 min) increased $\dot{V}O_{2\,max}$ associated with muscle increased mitochondrial enzymes activities (citrate synthase and cytochrome c oxidase), mRNA levels of mitochondrial genes (e.g. COX4) and genes involved in mitochondrial biogenesis (PGC-1α,NRF-1,TFAM) in skeletal muscle. These results suggested that aerobic exercise could induce *de novo* mitochondrial biogenesis and improve mitochondrial functions during aging. Indeed, Lanza et al. (2008) demonstrated in older trained people (performing at least one hour of cycling or running 6 days per week over the past 4 years) increases in mitochondrial ATP production rate, citrate synthase activity, PGC1-α, NrF-1 and Tfam muscle protein content, and mtDNA abundance. Moreover, an increased Sirt 3 protein content (known to stimulate PGC-1α) was also found. Recently, Safdar et al. (2010) confirmed such results and showed that they are associated with functional improvements (increase in maximal isometric strength, decrease in time to perform

the 30-feet walk test and stair climb test). Moreover, they also found that physical activity in older people increased complex IV activity and COX subunits-I and II protein content in skeletal muscle. More recently, Koltai et al. (2012) showed in old trained rats (6 weeks of treadmill training at 60% $\dot{V}O_{2\,max}$, one hour per day) that increased mitochondriobiogenesis (attested by increased PGC-1α, SDH and COX 4 muscle protein content and increased mtDNA abundance) is driven by an increase in Sirt 1 activity and AMPK phosphorylation. Moreover, these authors found that aerobic exercise is able to restore mitochondrial dynamics (fusion and fission) to similar levels of those observed in young rats (as attested by measurement of Mitofusin 1, fission protein-1 and Lon protease protein content) which would reflect a reduction of impaired mitochondria. Konopka et al. (2013) confirmed these results in older people after an aerobic training (4 sessions of 45 min per week at 80% heart rate reserve). Indeed, trained elderly presented increased Mitofusin 1 and 2, fission protein-1 as well as PGC-1α and citrate synthase muscle protein content associated with an increase in $\dot{V}O_{2\,max}$ and CSA.

Because resistance training is usually not associated with mitochondrial functions improvements, very few studies are available on this topic. However, Parise et al. (2005) found in older people after twelve weeks of whole body resistance training (3 sessions per week, 80% of 1RM) an increase in complex IV activity, reflecting ETC improvements. Moreover, Luo et al. (2013) found in older rats an increased AMPK phosphorylation associated with an increased cytochrome C mitochondrial protein content in skeletal muscle after a nine weeks resistance training. However, in both studies physical parameter were not measured.

1.5. Exercise during aging would restore a young redox status

As well reviewed by Ji (2001), although aged muscles demonstrated higher levels of ROS generation when they are subjected to an acute bout of exercise at a given workload, aerobic or resistance training can decrease oxidative damage (Radák et al. 2002; Lambertucci et al. 2007). This beneficial effect is not specific to skeletal muscle since it can be found in others tissue such as heart (Fiebig et al. 1996) and liver (Nakamoto et al. 2007).

Numerous studies examined the effect of aerobic exercise on OS in skeletal muscle in humans and rats. However, here we will focus only on those in which trained elderly or old animals were compared to old and young sedentary or trained subjects and animals. Thanks to this approach, we will show that aerobic exercise is able to restore a “young redox status”. All the parameters which will be presented were observed measured skeletal muscle and were

always enhanced compared to older sedentary.

Moderate intensive endurance training (at least 5 one-hour treadmill sessions per week at 55-65% of $\dot{V}O_{2\,max}$ or VMA at least during 6 weeks, or life-long voluntary exercise with running wheel in animals or modest recreational activities such as golfing, tennis and/or cycling at least 3 times per week in humans) in elderly people or old rats was associated with a restoration of oxidative damage (lipid peroxidation, carbonylated and nitrozilated proteins, DNA oxidation) to similar levels than those observed in young people (Safdar, Hamadeh, et al. 2010) or rats (Radák et al. 2002; Rosa & Silva 2005; Lambertucci et al. 2007; Kim et al. 2008; Koltai et al. 2010; Wohlgemuth et al. 2010). This fact can be explained by several mechanisms. An increase in repair systems as found by Radak et al. (2002) which showed decreased 8-OHdG nuclear content (similar to young rats) associated with an increase in 8-OHdG repair system enzyme activity in response to aerobic training. Moreover, decrease muscle protein content and/or activity of free radicals sources (e.g. XO, NOS) has been shown to be involved in restoring young oxidative damage levels after endurance training. Thus, Lambertucci et al. (2007) found that endurance training in old rats reduced xanthine oxidase activity to similar levels than those observed in young rats, associated with comparable levels of lipid peroxidation. In the same way, endurance training in older people was able to maintain comparable nNOS muscle protein content to young sedentary people associated with similar muscle content of nitrozilated proteins. Increase antioxidant enzymes activities to rise activities observed in young people is also involved in restoring a young redox status in response to aerobic training during aging. Indeed, older people engaged in aerobic exercise presented similar Mn-SOD and total SOD activities compared to young people associated with comparable nitrozilated proteins levels. Regardless of whether the activity of antioxidant enzymes is increased or decreased during aging, endurance training restore similar levels to those observed in younger. Indeed, in the study of Lambertucci et al (2007) aging was associated with increased antioxidant enzymes activities and endurance training reduced these latter to similar levels than younger, whereas it happened the contrary in the study of Safdar et al. (2010). In all the studies presented, when measured, $\dot{V}O_{2\,max}$ or VMA were improved by the proposed training protocol.

In regards to resistance training, it is not possible to conclude to the same phenomenon because to our knowledge, no studies compared old trained people to sedentary old and young people. However, resistance training in older humans (2-3 sessions per week during at least twelve weeks with exercises at 80% of 1RM) reduced 8-OHdG/creatinine ratio in urine (reflect of muscle oxidative DNA damage) surely due to an increase in CuZnSOD and

catalase activity in skeletal muscle (Parise, Phillips, et al. 2005; Parise, Brose, et al. 2005; Tarnopolsky et al. 2007). In those studies, resistance training was able to increase the 1RM.

Finally, exercise appears to be the best countermeasure against sarcopenia because it can act on all the deleterious effect induced by aging and improve at the same time muscle mass, strength and physical performance. Neuromuscular adaptations were beyond the scope of this part, however, they have been well reviewed by (Aagaard et al. 2010). As presented, resistance training leads to the most profound gains in strength and muscle mass while aerobic training leads to enhance $\dot{V}O_{2\,max}$ and endurance capacity. Perform resistance training cycles and endurance training separately appears to be the best solution to combat sarcopenia.

2. Alternative strategies to exercise for fighting sarcopenia

Although exercise training is highly effective in counteracting age-related muscle loss, the large scale implementation of such intervention is hampered by the lack of motivation of most persons. In addition, many elderlies are non-ambulatory or have co-morbidities such as moderate to severe osteoarthritis (Bennell & Hinman 2011) or certain forms of unstable cardiovascular disease that would preclude participation in resistance training exercises (Williams et al. 2007). To overcome such barriers, developing alternative therapies for the prevention and treatment of sarcopenia such as antioxidant strategies (e.g. antioxidants supplementation, pharmacological inhibitors of pro-oxidant enzymes), hormones replacement-therapies (e.g. growth hormone, testosterone) or pharmacological treatment (angiotensin-converting-enzyme inhibitor, statins, myostatin inhibitors are important. Here, we will focus only on antioxidant strategies and hormones replacement-therapies (others strategies have been well reviewed by Sanchis-Gomar et al. 2011; Maggio et al. 2013; Morley & Malmstrom 2013).

2.1. Possible antioxidant strategies to attenuate sarcopenia

In the literature, different kinds of antioxidant strategies are presented. The first will aim to directly scavenge the RONS presented in the organisms by supplementation with one antioxidant or a cocktail of various antioxidants such as vitamin C, vitamin E and carotenoids, or supplementation with natural compounds (which can be modified to increase their bioavailability) such as resveratrol. The second will directly target RONS sources with pharmaceutical products such as allopurinol which is an inhibitor of xanthine oxidase. The last strategy will consist in making a supplementation with precursors of the synthesis of antioxidant molecules such as precursors of GSH synthesis.

Usually, studies showed that these strategies are able to decrease age-related oxidative damage due to an increase in antioxidant defenses in skeletal muscle. Indeed, Kumaran et al. (2004) found in old rats that orally supplementation with a mix of L-carnitine (300 mg/kg body weight per day) and DL-α lipoic acid (100 mg/kg body weight in alkaline saline per day) during 30 days, was able to reverse the muscle age-related decline of GHS/GSSG ratio. The beneficial effect was explained by the increase of Gpx, GR and G6PDH activities to similar levels than observed in young rats (old control rats presented decreased activities of theses antioxidant enzymes). Similar results were found in heart. Analogous results were shown by this group in response to epigallocatechin-3-gallate supplementation (EGCG) a key component of green tea catechins (100 mg/kg of body weight per day by oral gavage for 30 days). They also found that EGCG was able to reverse the age-related decrease in GSH/GSSG ratio, and Gpx, GR and G6PDH activities. Moreover, these authors showed that EGCG leads to reverse the age-related decrease in total SOD and Cat activities in skeletal muscle. These different effects were associated with decreased lipid peroxidation and protein carbonylation. Recently, Laurent et al. (2012) explored the effect of 30-day oral supplementation with a moderate dose of a red grape polyphenol extract (RGPE) on major systems of RONS production (i.e. NOX) and their consequences on OS, mitochondriogenesis and muscle metabolism in aged rats. They found that this strategy reversed the age-related decline of total SOD and Cat activities but failed to showed beneficial effects on lipid peroxidation and protein oxidation. Note that RONS production by NOX activity was similar between young and old, control and treated animals. Moreover, an increase in PGC-1α muscle protein content was observed but was not associated with mitochondrial biogenesis as shown by the absence of increase in citrate synthase activity. Unfortunately, in these three aforementioned studies no data such muscle mass was measured to prove that such changes in oxidative parameters had reversed or limited sarcopenia. It appears very important to do it because as it will be presented, reduce OS with these strategies is not always associated with sarcopenia attenuation. Indeed, old mice receiving a diet supplemented with resveratrol (0,05 % of the total diet) during 10 months presented decreased H_2O_2 muscle content and reduced lipid peroxidation levels associated with an increase in Mn-SOD activities. These parameters were comparable to those observed in younger mice. However, muscle weight (gastrocnemius and plantaris) and functions were not improved (Jackson et al. 2011). Finally, although improving oxidative damage strategies based on natural compounds appears to not attenuate sarcopenia.

Although they have not made a direct antioxidant supplementation, Semba et al (2003) showed in almost 700 non-disabled to severely disabled community-dwelling women aged 70 to 79 years old that higher carotenoid and alpha-tocopherol plasma concentrations were independently associated with higher strength measures. Recently, Saito et al (2012) published a similar positive relation in an analogous population between plasma vitamin C levels and walking speed and hand grip strength. These results suggest that antioxidant supplementation would be efficient in combating sarcopenia. However until known studies realizing such treatment in humans and animals failed to improve muscle mass, strength or physical performance or did not measure these latter. Indeed, although an antioxidant supplementation in old rats with an antioxidant cocktail during 7 weeks (vitamin E, vitamin A, zinc, and selenium) was able to improve the ability of leucine to stimulate protein synthesis in muscles of old rats, no clear effect on muscle mass was observed (Marzani et al. 2008). Antioxidant supplementation was probably not long enough. This study highlighted that an optimal redox status would be an important in protein synthesis. Recently, Nalbant et al. (2009) and Bobeuf et al. (2011) in older people receiving respectively only vitamin E or an antioxidant cocktail (vitamin C and vitamin E) during 6 months failed to show improvement in physical performance and muscle strength. Finally, antioxidant supplementation alone appears to not be efficient in fighting sarcopenia.

Evidence that antioxidant strategies can be a good option to fight sarcopenia was recently brought by Sinha-Hikim et al. (2013). In this study, they supplemented old mice from 18 months old to 23 months old with a GSH precursor cocktail containing L-cystine, selenomethionine and L-glutamine. Old control animals presented gastrocnemius atrophy (attested by weight and CSA) associated with increased OS and decreased antioxidant enzymes activities, exacerbated apoptosis, reduced regenerative potential of skeletal muscle and maybe impaired protein turnover (supposed by a decreased phosphorylation of Akt). On the other hand, old rats treated with this GSH precursor cocktail presented an increased GSH/GSSG ratio associated with an increase in G6PDH muscle protein content. Moreover, the age-related decline in SOD activity was totally reversed as well as lipid peroxidation. These beneficial effects on OS were concomitant to an improved regenerative potential of skeletal muscle (attested by an up-regulation of the principal compounds of the Notch signaling), a decreased apoptosis index and an increased Akt phosphorylation. Finally, old treated mice presented a higher muscle mass measured through a higher muscle weight and CSA. Recently, although they did not directly treat older people with allopurinol (pharmaceutical inhibitor of xanthine oxidase), Beveridge et al. (2013) showed in a

retrospective observational study that allopurinol use is associated with greater functional gains in older rehabilitation patients. Moreover, Derbre et al. (2012) showed in young animal that allopurinol protected against muscle atrophy induced by hind limbs suspension. These data suggested that allopurinol could be a good intervention to prevent sarcopenia. However it seems that it has been never studied.

2.2. Exercise and antioxidant supplementation at old age

This part was extracted and modified from the review "Exercise and antioxidant supplements in the elderly" written by Gomez-Cabrera, Ferrando, ***Brioche****, Sanchis-Gomar, Viña and published in Journal of Sport and Health Science in 2013. A full version is available in the annex part of this manuscript.*

The beneficial effect of physical activity for the promotion of health and curing of diseases among individuals of all ages is beyond all doubt. Strong scientific evidences link physical activity to several benefits, including the promotion of health span and not only of lifespan. Although physical activity has many well-established health benefits (Vina et al. 2012), aging and strenuous exercise are associated with increased free radical generation in the skeletal muscle (Ji 2001). Thus, whether exercise would worsen the skeletal muscle OS in aged population has been an object of debate. Research evidence indicates that senescent organisms are more susceptible to OS during exercise because of the age-related ultrastructural and biochemical changes that facilitate ROS generation (Ji 2001). Aging also increases the incidence of muscle injury, and the inflammatory response can subject senescent muscle to further OS. Furthermore, muscle repair and regeneration capacity is reduced at old age that could potentially enhance the cellular oxidative damage (Ji 2001). Thus, several researchers consider that dietary antioxidant supplementation should be beneficial in the old physically active population (Bobeuf et al. 2011). Recent studies suggested a beneficial relationship between antioxidant vitamin (e.g., vitamin C) intake and physical performance in elderly people (Saito et al. 2012). It has been shown that intake of resveratrol, together with habitual exercise, is beneficial for suppressing the aging-related decline in physical performance (Ryan et al. 2010). Moreover, it has been shown that antioxidant supplementation improves indices of OS associated with repetitive loading exercise and aging and improves the positive work output of muscles in aged rodents (Ryan et al. 2010). Bobeuf et al. (2010) found that six months of resistance training (3 days per week, 80% 1RM) combined with antioxidant supplementation significantly increased fat-free mass in older adults. However, these results have not been confirmed by other studies. Nalbant et al. (2009)

found that six months of vitamin E supplementation had no additive effect beyond that of aerobic training (3 days per week, 70% heart rate reserve) on indices of physical performance and body composition in older sedentary adults. Regarding bone density it has been shown that combination of resistance training with antioxidant vitamins supplementation does not seem to produce synergistic effects on the prevention of osteoporosis (Christen 1994). The convenience of supplementing with antioxidant vitamins in the old sport population is nowadays, as in the young population, an object of debate. In fact training studies conducted in young people to determine whether antioxidant vitamins improve exercise performance have generally shown that supplementation is useless (Gey et al. 1970; Yfanti et al. 2010; Maughan 1999; Keren & Epstein 1980; Theodorou et al. 2011) or even negative (Gomez-Cabrera, Ristow, et al. 2012). Several studies suggest that antioxidants may have detrimental effects on performance (Sharman et al. 1971; Malm et al. 1997; Malm et al. 1996; Marshall et al. 2002). Our group has found that vitamin C supplementation decreases training efficiency because it prevents exercise-induced mitochondrial biogenesis (Gomez-Cabrera, Domenech, Romagnoli, et al. 2008). These results have been confirmed by other research groups (Kang et al. 2009; Ristow et al. 2009). A large proportion of athletes, including elite athletes, take vitamin supplements, often large doses, seeking their beneficial effects on performance (Sobal & Marquart 1994). The complete lack of any positive effect of antioxidant supplementation on physiologic and biochemical outcomes consistently found in human and animal studies raises questions about the validity of using oral antioxidant supplementation in the sport population (Gomez-Cabrera, Ristow, et al. 2012). On the other hand, Richardson's research group identified a clinically significant paradoxical cardiovascular response to exercise training and antioxidant supplementation in the elderly (Wray et al. 2009). Antioxidant administration, after exercise training, blunted training-induced reduction in blood pressure as well as the exercise-induced improvements in flow-mediated vasodilation. The paradoxical effects of these interventions suggest a need for caution when exercise and acute antioxidant supplementation are combined in elderly mildly hypertensive individuals. Moreover, previous reports showed that long-term vitamin E supplementation may increase the risk for heart failure in patients with vascular disease or diabetes mellitus (Lonn et al. 2005). In another report, Bjelakovic et al. (2007) looked at data from sixty-seven studies on antioxidant supplements and they concluded that high beta carotene, vitamin A, and vitamin E supplementation seemed to increase the risk of death. These data show that we must be cautious about the use of antioxidants and they underscore the need for more studies on doses

to administrate, the perfect time course for the administration and the choice of the antioxidant strategy to adopt in each situation.

Finally, the paradoxical effects of antioxidant supplementation, when combined with exercise training, reveal an intriguing, but complex, relationship between aging, exercise, and OS. More research for a better clarification of the field is required. As very few studies have shown beneficial effects of antioxidant strategies on sarcopenia, it seems imperative to consider other strategies such as hormones replacement-therapies for fighting sarcopenia.

2.3. Hormones replacement-therapies as a possible strategy

There is evidence that hormones in particular testosterone, dehydroepiandrosterone Sulphate (DHEA which after extraglandular metabolism lead to physiologically active testosterone) and growth hormone (GH) whose levels decrease with age, exert an important role in the age-related onset of sarcopenia (Sakuma & Yamaguchi 2012; Giannoulis et al. 2012; Maggio et al. 2013). Consequently, numerous studies try to reverse sarcopenia with these latter. A particular attention will be brought to GH because it was used in a study of this work.

After reviewing more than 150 studies, Baker et al. (2011) conclude that DHEA replacement therapy alone failed to increase muscle mass or strength in older persons. For instance, Percheron et al. (2003) tested on 280 healthy ambulatory and independent men and women (aged 60 to 80 years), if 1-year administration of a replacement dose of DHEA (50 mg per day, orally administered) could have a beneficial influence on several determinants of the muscle strength and body composition. Although this treatment restores DHEA serum concentrations to the normal range for young adults (aged 20-50 years), no positive effect was observed either on muscle strength or in muscle and fat cross-sectional areas. However, beneficial effects of DHEA treatment have been found when it is combined with others strategies (Baker et al. 2011; Maggio et al. 2013). Indeed, in a recent study, where elderly people were receiving DHEA and vitamin D for 6 months (50mg per day), Kenny et al. (2010) observed a slightly improvement in the short physical performance battery (SPPB). On the other hand, Villareal & Holloszy (2006) provided evidence that DHEA replacement has the beneficial effect of enhancing the increases in muscle mass and strength induced by heavy resistance exercise in elderly individuals. However, more studies are needed to confirm these results.

In 2006, the findings from 11 randomized controlled trial were examined using the methods of meta-analysis to determine whether androgen treatment (testosterone or its more potent sub-product 5 α-dihydrotestosterone) increased strength in men aged 65 years old and older (Ottenbacher et al. 2006). This meta-analysis was recently completed by Maggio et al. (2013) which reviewed the most recent randomized controlled trial done. These authors concluded that testosterone or 5 α-dihydrotestosterone treatment is useful to increase muscle mass, strength and physical performance (Ottenbacher et al. 2006; Maggio et al. 2013). The most convincing and complete data come from the Testosterone in Older Men with Mobility Limitations (TOM) Trial realized by Travison et al. (2011). The aim of this placebo-controlled randomized trial was to determine whether testosterone therapy (10 g testosterone gel daily for 6 months) in community-dwelling men (age of 74 years) affected by severe limitation in mobility improves muscle strength and physical function. Muscle strength was assessed by leg-press and chest-press strength. Physical function was evaluated using a 12-step stair-climb and 40 meters walk tests. Muscle fatigue was also assessed by trials of lifting and lowering a basket holding a weight equivalent to 15% body weight. Finally, lean body mass was determined by DXA. All these parameters were enhanced by this treatment and were associated with increases in serum total and free testosterone. However, adverse cardiovascular events occurred in more men receiving testosterone compared to men receiving placebo leading to stop the study. This study highlighted that despite numerous significant beneficial effects induced by testosterone treatment among elderly men, more studies are needed to find the perfect treatment. Currently, intermittent treatments and/or treatments associated with 5 α-reductase inhibitors (to avoid prostate risk) are new approaches tested to decrease adverse effects of testosterone.

Different mechanisms can explain the beneficial effects of testosterone. Testosterone is known to stimulate muscle protein synthesis, improve recycling of intracellular amino acids, decrease protein breakdown rate, and enhanced neuromuscular function (increase motoneurons activity) (Dubois et al. 2012). Testosterone also promotes satellite cells activation and inhibits their differentiation into adipocytes *via* an androgen receptor-mediated pathway (Grossmann 2011). Testosterone treatment is also associated with elevation in hemoglobin which can be considered an additional mechanism by which this hormone ameliorates muscle oxygenation and function (Fernández-Balsells et al. 2010). Moreover, testosterone seems to have anti-inflammatory effects since it can reduce the plasma concentration of TNF-α and several interleukins (Malkin et al. 2004). Recently it has been shown that testosterone is effective to reverse sarcopenia in rodents (Kovacheva et al. 2010).

These authors showed that testosterone decreased lipid peroxidation and apoptosis and explain this fact by the concomitant increase in G6PDH muscle protein content. Moreover, testosterone treatment led to satellite cells activation though the Notch signaling pathway due to myostatin inhibition and Akt activation.

Growth hormone is a single-chain peptide of 191 amino acids produced and secreted mainly by the somatotrope cells of the anterior pituitary gland. GH coordinates the postnatal growth of multiple target tissues, including skeletal muscle (Florini et al. 1996). GH secretion occurs in a pulsatile manner with a major surge at the onset of slow-wave sleep and less conspicuous secretory episodes a few hours after meals (Ho et al. 1988) and is controlled by the actions of two hypothalamic factors, GH-releasing hormone (GHRH), which stimulates GH secretion, and somatostatin, which inhibits GH secretion (Giannoulis et al. 2012). The secretion of GH is maximal at puberty accompanied by very high circulating IGF-I levels (Moran et al. 2002), with a gradual decline during adulthood. Indeed, circulating GH levels decline progressively after 30 years of age at a rate of ~1% per year. In aged men, daily GH secretion is 5- to 20-fold lower than that in young adults (Ryall et al. 2008). Moreover, Veldhuis et al. (1995) found a decrease in GH secretory burst amplitude mass with age (maximal rate of GH secretion attained within a release episode). The age-dependent decline in GH secretion is secondary to a decrease in GHRH and to an increase in somatostatin secretion (Kelijman 1991).

The effects of GH administration in elderly people on muscle mass, strength and physical performance are still under debate (Giannoulis et al. 2012). Some groups demonstrated an improvement in strength after short and long-term administration (3–11 months) of GH (Welle et al. 1996; Brill 2002; Blackman et al. 2002). For instance, Welle et al. (1996) found in healthy subjects over 60 years old that GH treatment for 3 months (0.03 mg per kg of body weight subcutaneously, 3 times per week) increased lean body mass, muscle mass, and thigh strength. Data in the same way were published by Blackman et al. (2002) in 26-week randomized, double-blind, placebo-controlled parallel-group trial in healthy, ambulatory, community-dwelling US and men aged 65 to 88 years old receiving 20 µg/kg of body weight subcutaneously 3 times per week. Treated men presented a fat mass decrease associated with a lean mass increase (which was higher to another group receiving testosterone). Furthermore, men's $\dot{V}O_{2\ \max}$ increased with GH and was directly related to changes in lean body mass. Unfortunately, some adverse effects such as arthralgia were more common in men taking GH. Interestingly, it has been shown in older men that GH therapy led to a substantial increase in MHC 2X isoform (Lange et al. 2002). In contrast, others groups

have found that muscle strength or muscle mass did not improve on therapy with GH in the elderly (Giannoulis et al. 2012). Several reasons may underlie the lack of effectiveness of GH treatment in particular failure of exogenous GH treatment to mimic the pulsatile pattern of natural GH secretion (Sakuma & Yamaguchi 2012). In addition, reduced mRNA levels of the GH receptor in skeletal muscle have been observed in older *versus* younger healthy men (Léger et al. 2008). In animal models, beneficial effects were also found in particular when using recombinant human GH. Indeed, Andersen et al. (2000) observed in old rat treated with GH (2,7 mg per kg per day during 12 weeks) an increase in calf musculature maximal tetanic tension (soleus, plantaris, gastrocnemius, tibialis anterior, EDL) associated with muscle hypertrophy (assessed by muscle weight and volume) surely due to the concomitant increased protein synthesis. In the same way, Castillo et al. (2005) showed that GH treatment during 4 weeks (2 mg/kg per day diluted in saline solution, divided into two subcutaneous injections, at 10:00 and 17:00 h) increased lean mass and decreased fat mass. However, others did not find such beneficial effects may be due to a shorter treatment duration, different dose or the source of GH (e.g. recombinant porcine GH) (Marzetti, Groban, et al. 2008).

Surprisingly, molecular mechanisms by which GH would increase muscle mass, strength and maximal oxygen consumption in the elderly and older animals have been poorly studied in skeletal muscle. However, data have been provided in other aged tissues, contexts or in young people and animals. Thus, chronic GH administration has been shown to reduce OS by increasing the concentration of glutathione in central nervous system and liver in long-living dwarf mice (Brown-Borg & Rakoczy 2003). This effect could be driven by an up-regulation of G6PDH (activity and or expression) since it has been shown that GH is able to up-regulated G6PDH *in vitro* (Gevers et al. 1996) and in rat liver (Gumaa et al. 1969) but to our knowledge this effect has never been shown in skeletal muscle. Furthermore, an anti-apoptotic effect in the heart of senescence-accelerated mice have been supposed since RNA level of TNF-α and several pro-apoptotic effector such as BAX and Bad were decreased in response to GH treatment (30 days, 2mg/kg per day) (Forman et al. 2009). This effect was confirmed in atrophied rat with heart failure treated with GH (1mg/kg per day) where apoptosis index was decreased in soleus muscle (Vescovo et al. 2005). In the same study, they found that GH treatment was able to enhance markers of mitochondriogenensis (PGC-1α and cytochrome c) in soleus muscle. On the other hand, it has been shown in hepatoma cells culture that activation of protein synthesis by GH requires signaling through mTOR (Hayashi & Proud 2007). Also, effects of GH are known to be driven by IGF-1 which can be produced in either the liver or in muscle. Different isoforms of IGF-1 have diverse effects. Liver-

derived IGF-1 appears to predominantly increase muscle mass by improving protein synthesis, whereas muscle-derived IGF-1 has effects on the development of satellite cells and on maintenance of neuromuscular function (Perrini et al. 2010). On the other hand, it has been shown that IGF-1 is able to slow protein breakdown (Dubois et al. 2012) and decrease apoptosis and OS *in vitro* (Yang et al. 2010). All these mechanisms need to be confirmed in a sarcopenic context.

Nowadays, it appears that although hormone replacement therapies notably testosterone and GH are useful in improving muscle mass in the elderly with limited mobility, more studies are needed to continue to explore others parameters of the different treatments such as doses, duration and periodicity (intermittent *versus* continuous) to avoid adverse effects. In GH treatment, try treatments mimicking its pulsatile secretion. Moreover, by understanding by which mechanisms hormones act in older animals, it could be possible to find new molecules to target in a sarcopenia context and more generally to fight muscle disuse in various situation (cachexia, immobilization), or to improve muscle hypertrophy in an exercise context.

As previously exposed, effective strategies to attenuate sarcopenia are able to improve redox status, mitochondrial functions and protein synthesis. They can also decrease apoptosis or activate cell proliferation (notably satellite cells). All these strategies are known to up-regulate the glucose-6-phosphate dehydrogenase (G6PDH) which is known to be involved in these different mechanisms (as it will be presented). Consequently, G6PDH would be a potential target in strategies against sarcopenia. Moreover, DHEA known to inhibit G6PDH *in vitro* (Tian et al. 1998), failed to attenuate sarcopenia when supplemented alone. On the contrary, beneficial effects were obtained with DHEA in combination with others strategies (i.e. exercise and vitamin D) which are recognized to increase G6PDH activity (i.e. exercise and vitamin D; Barakat et al. 1989; Stanton 2012) . Thus, in the following section, it will be presented with more details the reasons why G6PDH could be a new potential target to fight sarcopenia.

3. The Glucose-6-Phosphate Dehydrogenase as potential target to fight sarcopenia

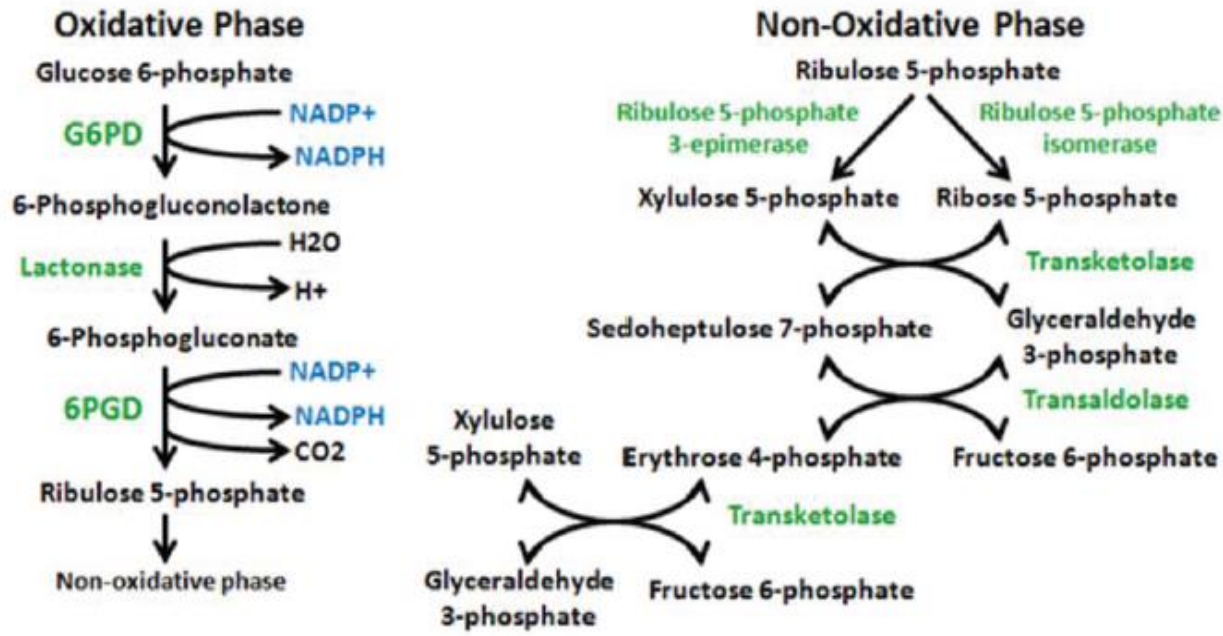
The glucose-6-phosphate dehydrogenase (G6PDH) was first described in 1931 (Kornberg et al. 1955), and the classic technique for measuring its activity is basically the same as used today (measure the rate of increase of absorbance at 340 nm from the conversion of NADP to NADPH by G6PDH). Most studies have since focused on G6PDH deficiency (which is associated with hemolysis after eating certain foods or taking certain medications), and lipid metabolism. G6PDH deficiency is the most common gene mutation in the world, and the numerous mutations have been classified by the World Health Organization (Nkhoma et al. 2009) according to the activity as follows: class I is < 1% of wild-type activity; class II is <10%; class III is 10–60%; class IV is 60–90% (considered normal activity); and class V is > 110%. It is estimated that at least 400 million people worldwide are G6PDH deficient and most are class III. During the last decade, studies have started to explore its role in diabetes (Park et al. 2005a), heart failure (Assad et al. 2011) and cancer (Kuo et al. 2000). However, it is now clear that G6PDH is a critical metabolic enzyme under complex control that resides at the center of an essential metabolic nexus that affects many physiological processes. Surprisingly, its role in skeletal muscle have been poorly studied whereas several clinical cases of rhabdomyolysis due to G6PDH deficiency have been reported more than fifteen years ago (Kimmick & Owen 1996). Moreover, numerous studies have shown since the eighties that dysregulation of its activity is associated with myopathies (Elias & Meijer 1983; Meijer & Elias 1984). Thus, it appears important to study its implication in skeletal muscle physiology and physio-pathology. Here, we will present data showing that down-regulation of G6PDH would be involved in sarcopenia through several mechanism such as decreased antioxidant capacity. On the other hand, we will provide data suggesting that the up-regulation of G6PDH would be a good strategy to combat sarcopenia. However, we will first remember it functioning.

3.1. G6PDH biochemistry and regulation in skeletal muscle

G6PDH controls the entry of glucose-6-phosphate (G6P) into the pentose phosphate pathway (PPP) also known as hexose monophosphate shunt. Figure 16 shows an initial irreversible oxidative stage of which G6PDH is the first and rate-limiting enzyme and a reversible nonoxidative stage in which transketolase and transaldolase are the key enzymes. The major products of the PPP are ribose-5-phosphate (R5P) and nicotinamide adenine

dinucleotide phosphate (NADPH) generated from NADP by G6PDH and the next enzyme in the pathway 6-phosphogluconate dehydrogenase (PGD). In the following paragraphs it will be exposed why through NADPH and R5P, G6PDH may be involved in sarcopenia and why enhancing their production by G6PDH would help to combat sarcopenia. Not long ago, G6PDH was only described as the principal source of NADPH in the cytosol. However, it has been recently shown that G6PDH is present in the mitochondria of skeletal muscle cells and provides NADPH like isocitrate dehydrogenase (ICDH), malic enzyme (ME) and glutamate dehydrogenase (GDH) which were originally described as the principal sources of NADPH in mitochondria. Thus, NADPH is mainly produced by five enzymes in mammalian cells, G6PDH, 6-PGD, ICDH, ME and GDH. All have been studied extensively and play critical cellular roles. However, G6PDH appears to be of unique importance to many cellular processes that use NADPH, since its inhibition lowers NADPH levels which are not maintained at normal levels by the other enzymes providing NADPH (Stanton 2012; Hecker & Leopold 2013).

Figure 16. The penthose phosphate pathway (extracted from Hecker & Leopold 2013).



It has been traditionally taught that G6PDH is regulated by the NADPH/NADP ratio so that as the ratio decreases, activity increases to provide more NADPH. Indeed, G6PDH is activated following exposure of cells to various extracellular oxidants (Kletzien et al. 1994) that lead to decrease in the level of NADPH. Regulation by the NADPH/NADP ratio has been clearly demonstrated in vitro (Holten et al. 1976), but not in vivo. G6PDH is highly regulated

at the transcriptional, translational, and post-translational level, and intracellular location. G6PDH is the downstream target of many molecules (see table 11) in particular growth factors and their downstream. In in skeletal muscle of mice, it has been found that testosterone treatments known to activate the PI3K/Akt/mTOR pathway is able to increase G6PDH activity and protein content associated with muscle hypertrophy (Max 1984; Kovacheva et al. 2010). Aerobic training has been also found to increase G6PDH activity in rat skeletal muscle (Barakat et al. 1989). However, other factors also regulate G6PDH are resumed in the table 11. Interestingly, as shown in the table 11, G6PDH is mainly activated by growth factors suggesting a role in cell growing as it will be presented.

Table 11. Positive and Negative regulators of G6PDH (modified from Stanton 2012).

Positive regulators	Negative regulators
PDGF, EGF, VEGF, HGF	TNFα
Insulin	P38 MAPK
Benfotiamine (vitamin B1 analog)	P53
Vitamin D	AMPK
Testosterone ,Estrogens	Aldosterone
Growth Hormone	Angiotensine
Exercise	Arachidonic acid
PI3K, Akt, mTOR, p70S6K	cAMP
Nrf2	cAMP-dependent PKA
Src	
TIGAR	
Hsp27	
SREBP	
ATM	
Phospholipase C	
cGMP-dependent PKG	
Ras-GTPase	

Abbreviations: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; VEGF, vascular endothelial cell growth factor; HGF, hepatocyte growth factor; PI-3K, phosphatidylinositol-3-kinase; PKG, protein kinase G; mTOR, mammalian target of rapamycin; TIGAR, TP53-induced glycolysis and apoptosis regulator; Hsp27, heat-shock protein 27; ATM, ataxia telangiectasia mutated; SREBP, sterol-responsive element binding protein; PKA, protein kinase A; CREM, cyclic AMP response element modulator; Nrf2, nuclear-factor-E2-related factor; TNFa, tumor necrosis factor alpha; AMPK, 50 adenosine monophosphate-activated protein kinase

3.2. G6PDH, NADPH, antioxidant defenses and sarcopenia

Several antioxidant systems depend on the production of NADPH for proper function. The first is the glutathione system (see chapter 3) dependent on the production of reduced glutathione by glutathione reductase that depends on NADPH (M. D. Scott et al. 1993). Catalase does not need NADPH to convert hydrogen peroxide to water but has an allosteric binding site for NADPH that maintains catalase in its active conformation (M. D. Scott et al. 1993). Note that OS in erythrocyte from G6DP deficient people is generally attributed to a

decrease NADPH content leading to impaired glutathione recycling but the real mechanism is a dramatic decrease in catalase activity (M. D. Scott et al. 1993). There is a very strong positive correlation between G6PDH activity and catalase activity that is more elevated than the correlation between G6PDH activity and GSH content (M. D. Scott et al. 1993). Superoxide dismutase does not use NADPH to convert superoxide to hydrogen peroxide; however, if this is not adequately reduced chemically by catalase or glutathione, the increased hydrogen peroxide levels will quantitatively increase and inhibit the SOD activity (Stanton 2012). It has been shown in various studies that during sarcopenia and aging, decreased G6PDH activity and/or muscle protein content are associated with a depletion of GSH, an increase in the GSSG/GSH ratio associated with GR, Gpx, Catalase and SOD decreased activity (Kumaran et al. 2004; Kumaran et al. 2008; Kovacheva et al. 2010; Sinha-Hikim et al. 2013). These would explain the concomitant observed increase in lipid peroxidation and protein oxidation (Kumaran et al. 2004; Kumaran et al. 2008; Kovacheva et al. 2010; Sinha-Hikim et al. 2013). On the other hand, in response to different antioxidant strategies or testosterone treatment in rats, G6PDH protein content or activity was increased in skeletal muscle and a concomitant increase in GSH, GR, Gpx, Cat and SOD activities was observed leading to a reduce oxidative damage (Kumaran et al. 2004; Kumaran et al. 2008; Kovacheva et al. 2010; Sinha-Hikim et al. 2013). These results provided evidences that targeting G6PDH would be a good strategy to combat sarcopenia by restoring a young redox-status which is very important to reestablish protein synthesis and muscle regenerative potential (through satellite cells activation). As previously exposed, Kovacheva et al. (2010) published data in this way. Indeed, testosterone treatment in old mice was able to increase G6PDH muscle content associated with decreased lipid peroxidation and increased Akt phosphorylation and satellite cells activation. Finally, these mice presented muscle hypertrophy. Similar results have been published by Sinha-Hikim et al. (2013) in old mice in response to a treatment with a GSH precursor. In young animals, it has been shown that aerobic exercise can increase G6PDH activity in skeletal muscle and liver (Askeq et al. 1975). However, there is no data about exercise, sarcopenia and G6PDH. Although G6PDH supplies the antioxidant glutathione system with NADPH and appears to maintain Cat and SOD activity, the NADPH produced by G6PDH could be also used by several pro-oxidant systems such as NADPH oxidase (Nox), nitric oxide synthase (NOS), and xanthine oxidase which have been shown to dependent directly or not from NADPH (Porras et al. 1981; Xia et al. 1996; Babior 1999; Tsutsui et al. 2011). Although observed in specific condition such as heart failure (Hecker & Leopold 2013), this relation does not seem to occur in sarcopenia since this latter is associated

with a decreased G6PDH activity in skeletal muscle whereas RONS production increased through XO, NOS and NOX. Their NADPH source would be others enzymes or they would need a very small NADPH amount to work at their optimal level. Moreover, Braga et al. (2008) found a dramatic G6PDH protein content decrease in sarcopenic mice associated with an increased NOS protein content. In the same way, G6PDH overexpression in endothelial cell known to normally have a high XO activity, presented decreased RONS production by XO (Leopold et al. 2003).

3.3. G6PDH, apoptosis and sarcopenia

Various studies in cell culture have shown a direct negative relation between G6PDH activity and/or protein content and apoptosis (Salvemini et al. 1999; Tian & Braunstein 1999; Nutt et al. 2005; Fico et al. 2004). For instance, G6PDH-deleted embryonic stem cells a more sensitive to H_2O_2-induced apoptosis associated with GSH depletion and increased caspase 3 and 9 protein content as well as (Fico et al. 2004). On the other hand, Nutt et al. (2005) have shown that inhibition G6PDH by DHEA activated caspase 2 and promote oocyte apoptosis. In old rodents, in numerous studies, G6PDH decreased activity and/or protein content in skeletal muscle is associated with increased apoptosis and atrophy (Braga et al. 2008; Kovacheva et al. 2010; Sinha-Hikim et al. 2013). Moreover, Braga et al. (2008) confirmed in old mice that depletion in G6PDH protein content is associated with enhancement of caspase 2 and caspase 9 protein content in skeletal muscle. On the other hand, in response to different strategies to fight against sarcopenia, increased G6PDH activity is associated with decreased apoptosis and muscle hypertrophy (Kovacheva et al. 2010; Sinha-Hikim et al. 2013). This beneficial effect would pass through a link between Akt and G6PDH. Indeed, Akt is also known to have anti-apoptotic effects (Robey & Hay 2006). Moreover, in the aforementioned studies, in old muscle Akt and G6PDH protein were both decreased and associated with muscle atrophy (Kovacheva et al. 2010; Sinha-Hikim et al. 2013). Finally, decreased G6PDH muscle activity and/or protein content appeared to be involved in sarcopenia by promoting apoptosis through caspases activiation whereas up-regulation of these latter was associated with decreased apoptosis and muscle hypertrophy.

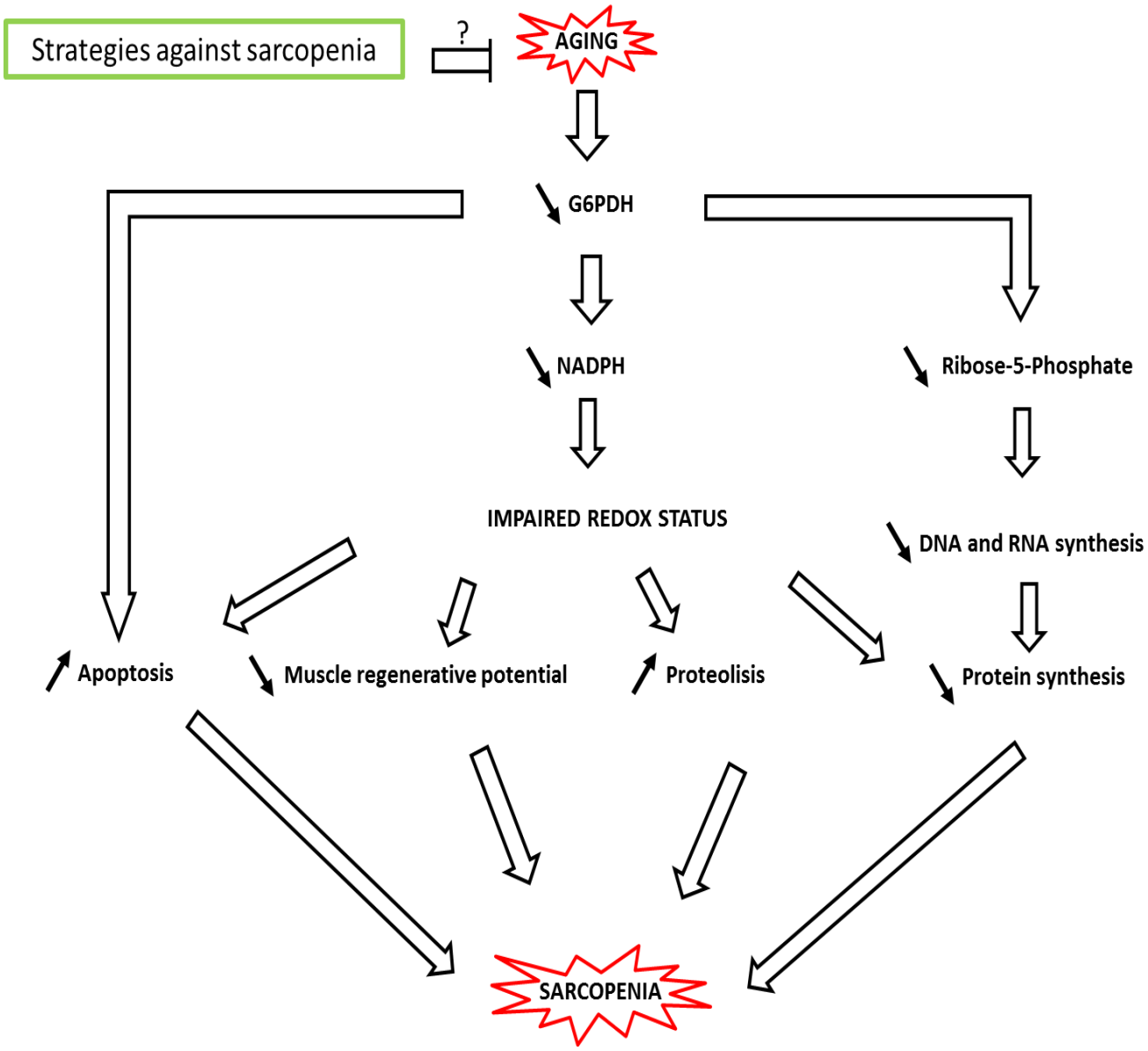
3.4. G6PDH, NADPH, ribose-5-phosphate and sarcopenia

G6PDH activity would have an important role in muscle hypertrophy and regeneration by acting on the potential proliferation of satellite cells, RNA and protein synthesis. Indeed, in various old works studying the muscle degeneration-regeneration cycle it, has been shown that during regeneration (known to involved satellite cells) G6PDH activity is dramatically increased (Wagner et al. 1977; Wagner et al. 1978) while protein synthesis and RNA synthesis were increased (Wagner et al. 1978). Moreover, inhibition of RNA and protein synthesis was associated with G6PDH inhibition (Wagner et al. 1978). Note that part of the G6PDH activity increase is due to the concomitant macrophage infiltration because they have a high G6PDH activity to provide NADPH to NOX to degrade necrotic tissue (Wagner et al. 1978). Increased quantities of RNA have been noted in a number of studies on muscle regeneration in response to pharmacological degeneration in rats (Susheela et al. 1966; Neerunjun & Dubowitz 1974). Thus, it was argued that G6PDH would play an important role in RNA and DNA synthesis since it is the rate limiting enzyme of the PPP which is the main pathway synthetizing R5P, an essential compound of nucleic acid. Through this role G6PDH would indirectly impact protein synthesis. These various hypotheses were confirmed by studies *in vitro* that have shown that overexpression of G6PDH accelerates proliferation of numerous cell lines associated with increased DNA and protein synthesis (Tian et al. 1998; Kuo et al. 2000). On the other hand, G6PDH deficient cells presented lower growth rate (Ho et al. 2000). An increased RONS production was observed in these cells suggesting an impaired redox status which would play an important role in the slower growth. Furthermore, inhibition of G6PDH caused cells to be more susceptible to the growth inhibitory effects of H_2O_2 due to NADPH decrease leading to reduce GSH content (Tian et al. 1998). Since, inhibition of G6PDH in cultured cells lead to decrease their proliferation due to a decreased protein and DNA synthesis associated with an impaired redox status, it could be hypothesized that G6PDH decrease (activity and protein content) observed in skeletal muscle during aging, would participate to reduce the regenerative capacity of skeletal muscle. On the other hand, increased G6PDH activity would improve this mechanism. Data in this way have been published by Kovacheva et al. (2010) which found old sarcopenic mice showed impaired satellite cells proliferation associated with decreased skeletal muscle G6PDH protein content and increased oxidative damage. Conversely treated mice with testosterone presented an increased G6PDH muscle protein content associated with satellite cells proliferation and decreased oxidative damage (Kovacheva et al. 2010). Furthermore, a hypothetical decreased

G6PDH activity into satellite cells during aging would participate in their lower capacity to proliferate and would make them more sensitive to oxidative stress. Moreover, based on the aforementioned studies, G6PDH decreased during aging would participate to decrease protein synthesis. Until now, only a decrease in Akt phosphorylation associated with decreased G6PDH activity and atrophy would support this hypothesis in skeletal muscle (Kovacheva et al. 2010; Sinha-Hikim et al. 2013).

Finally, decreased G6PDH activity and/or protein content in skeletal muscle observed during aging, would participate in sarcopenia by decreasing the antioxidant capacity attested by a decreased GSH content, catalase and SOD activities which are intimately linked. In consequence, the concomitant increased RONS production observed would damage cellular compounds in particular proteins which would impair the PI3K/Akt/mTOR pathway leading to decrease protein synthesis. There is no exiting data about G6PDH and proteolysis, however, by decreasing antioxidant defense, RONS would accumulate their self and promote the activation of several proteolysis pathway as exposed at the end of the chapter 3. On the other hand, the parallel decrease in Akt phosphorylation and G6PDH activity lead to activate apoptosis through caspases activation. Decrease in G6PDH activity would reduce the regenerative potential of skeletal muscle by limiting satellite proliferation. Activate G6PDH would restore an optimal redox status and reverse these adverse effects. All these mechanisms are resumed in the following figure (figure 17).

Figure 17. G6PDH-linked mechanisms possibly involved in sarcopenia.



4. Chapter 4 abstract

Exercise (aerobic and resistance) appears to be the perfect strategy against sarcopenia because it can lead to increase muscle mass, strength and physical performance (Pillard et al. 2011; Di Luigi et al. 2012; Wang & Bai 2012; Montero & Serra 2013). However, many elderlies are non-ambulatory or have co-morbidities that would preclude participation in training programs (Williams et al. 2007).

To overcome such barriers, alternatives strategies such as antioxidant strategies, in particular a GSH precursor cocktail (Sinha-Hikim et al. 2013), and hormone replacement therapies, in particular testosterone (Kovacheva et al. 2010; Travison et al. 2011) and growth hormone (Blackman et al. 2002; Andersen et al. 2000) have been tested in both humans and rodents. Like exercise, they presented beneficial effect on muscle mass, strength and physical performance.

These effective strategies against sarcopenia (including exercise), can improve protein turnover, reduce apoptosis, decrease mitochondrial dysfunction, activate mitochondriogenesis and muscle regeneration through satellite cells. Improvement of these mechanisms would be made possible thanks to a restoration of the redox homeostasis which appears as the common mechanism to all these different strategies.

The glucose-6-phosphate dehydrogenase (G6PDH) which is the rate limiting enzyme of the pentose phosphate pathways, is the main cellular source of NADPH which is necessary for an optimal functioning of antioxidants systems (glutathione system, catalase and indirectly superoxide dismutase). It seems that the restoration of redox homeostasis by the different effective strategies against sarcopenia involves an up-regulation of G6PDH muscle protein content and/or activity.

Moreover, data in *vitro* or in *vivo*, have suggested that G6PDH up-regulation would be involved in decreasing apoptosis, improving DNA, RNA and in fine protein synthesis and also muscle regeneration supposing that G6PDH would have a central role in the development of sarcopenia. However, these data need to be confirmed.

SYNTHESIS AND OBJECTIVES

The components of the sarcopenia definition are still in debate in the medical and scientific world. However, the different working groups agree on some points which can constitute the current consensus as follow. Sarcopenia is a geriatric syndrome initially characterized by a decrease in muscle mass that will get worse causing a deterioration in strength and physical performance (Muscaritoli et al. 2010; Cruz-Jentoft et al. 2010; Fielding et al. 2011; Morley et al. 2011). What people should be primarily target for a diagnosis? What would the standardized diagnostic? are questions still under debate.

The observed loss of muscle strength in sarcopenia is primarily due to muscle atrophy, while a decrease in the specific strength (i.e. the force generated relative to the surface of the fiber) is also involved. Muscle atrophy can be explained in part by the reduction in muscle protein synthesis and increased protein degradation *via* the ubiquitin-proteasome system and the calcium-dependent activation of proteases (i.e. calpains and caspases). Furthermore, exacerbation of myonuclei apoptosis results would decrease transcriptional efficiency and thus limit protein synthesis. This is probably worsened by alterations in aging muscle regeneration capacity with reduction of the incorporation of new nuclei and decrease in the pool of satellite cells and their capacity for proliferation and differentiation (in particular due to a less functional cellular and systemic environment). Moreover, the decrease in mitochondrial dynamics (biogenesis *vs* degradation *via* autophagy, fusion and fission) leads to the accumulation of defective mitochondria which then fall into a vicious circle, in which RONS production increases. All these mechanisms contribute to the onset of sarcopenia and are controlled by numerous signals such as decreased production of anabolic hormones (GH, IGF-1, testosterone, insulin). Links and interactions between these depleted hormones and the cellular dysfunctions cited earlier remain partly unknown. A potential candidate could be chronic oxidative stress, whose recent studies emphasize its involvement in sarcopenia.

Both in humans and animals, it has been showed that sarcopenic muscle exhibits increased RONS production (e.g. $O_2^{\bullet-}$ et H_2O_2) (Capel et al. 2004; Capel, Rimbert, et al. 2005; Capel, Demaison, et al. 2005; Chabi et al. 2008; Jackson et al. 2011; Andersson et al. 2011; Miller et al. 2012). This overproduction of RONS is mainly due to mitochondrial dysfunctions (Capel, Rimbert, et al. 2005; Chabi et al. 2008) and increased xanthine oxidase activity (Lambertucci et al. 2007; Ryan et al. 2011). RONS overproduction in sarcopenic muscle leads to an increase in oxidative damage to cellular components which reflect the inability of antioxidant systems to contain this overproduction and attest an imbalance of the "oxidants-antioxidants" balance leading to an impaired redox homeostasis, known as oxidative stress (Sies 1985; Jones 2006). *In vitro* studies showed that the oxidative stress in

muscle cells would reduce protein synthesis, cell regeneration capacity and stimulates proteolysis. Chronic oxidative stress observed in aged muscle could promote these mechanisms and lead to sarcopenia. Nevertheless, its implication on these cellular dysfunctions needs to be clearly demonstrated *in vivo*.

Exercise appears to be the perfect strategy against sarcopenia because it can lead to increase muscle mass, strength and physical performance (Pillard et al. 2011; Di Luigi et al. 2012; Wang & Bai 2012; Montero & Serra 2013). However, many elderlies are non-ambulatory or have co-morbidities that would preclude participation in training programs (Williams et al. 2007). To overcome such barriers, alternatives strategies such as antioxidant strategies, and hormone replacement therapies (testosterone and GH) have been tested in both old humans and rodents and showed an increase in muscle mass, strength and physical performance (Sinha-Hikim et al. 2013; Kovacheva et al. 2010; Travison et al. 2011; Blackman et al. 2002; Andersen et al. 2000). The effective strategies against sarcopenia can improve protein turnover, reduce apoptosis, improved mitochondrial functions and dynamics, and muscle regeneration. These improvements would be made possible thanks to a restoration of the redox homeostasis which appears as the common mechanism to all these different strategies.

It seems that the restoration of redox homeostasis by the different strategies against sarcopenia involves an up-regulation of G6PDH muscle protein content and/or activity which would supply NADPH to several antioxidant systems. Moreover, few data *in vitro* or *in vivo*, have suggested that G6PDH would play a central role in muscle mass regulation by increasing protein synthesis and/or decreasing proteolysis, decreasing apoptosis, improving cell proliferation and growth. Futhermore, Max (1984) and Kovacheva et al. (2010) have shown that in hypertrophic conditions there was an up-regulation of G6PDH. However, these data need to be confirmed.

In this context, this thesis will attempt to answer three general objectives. The first objective is to determine *in vivo* to what extent a pro-oxidant redox status due to aging within the muscle tissue may modulate signaling pathways involved in cellular mechanisms underlying sarcopenia. The second objective is to show that return to normal functioning of these signaling pathways requires a restoration the redox homeostasis. Finally, the third objective of this thesis is to identify actors and their possible mechanisms by which the redox homeostasis could be maintained.

The specific objectives of this thesis are:

- Determine whether the pro-oxidant redox status in skeletal muscle of aged rodents can modulate signaling pathways involved in protein synthesis and proteolysis but also in muscle regeneration and mitochondriogenesis leading to sarcopenia. We hypothesized in particular that oxidative stress would lead to a down-regulation of the PI3K/Akt/mTOR and PGC-1α/Tfam/Nrf-1 signaling pathways, and to an up-regulation of the ubiquitin proteasome system markers dependent as well as inhibitors of muscle regeneration (Study 1).

- Determine in which measures and by which mechanisms a treatment with growth hormone allows to prevent sarcopenia in older rodents. We make in particular two hypotheses.

  1) The GH *via* an increase in the IGF-1 circulating concentrations will allow restoring a normal functioning of the PI3K/Akt/mTOR signaling pathway while decreasing the expression several compounds of the ubiquitin proteasome dependent system and inhibitors of the muscle regeneration. A possible effect on the mitochondriogenesis is also envisaged (Study 1).

  2) These beneficial effects are made possible by an improved redox status in particular through overexpression of certain antioxidant enzymes (Study 1).

- Determine *in vivo* using a transgenic mouse model overexpressing Glucose-6-phosphate dehydrogenase (G6PDH), the roles of this enzyme in regulating body composition (muscle mass and fat mass) and its impacts on physical performances (muscle strength, maximal oxygen uptake and endurance capacity) (Study 2).

- Determine *in vivo* if the overexpression of G6PDH allows improved redox status in resting condition and protection against pro-oxidizing situations (exhaustive exercise and hyperoxia) (Study 3).

PERSONAL CONTRIBUTION

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Study 1: Growth hormone replacement therapy prevents sarcopenia by a dual mechanism: improvement of protein balance and of antioxidant defenses

Authors: Brioche T[1,2], Kireev RA[3], Cuesta S[3],Gratas-Delamarche A[2], Tresguerres JA[3], Gomez-Cabrera MC[1], and Viña J[1]

Affiliations:[1]Department of Physiology. University of Valencia. Fundacion Investigacion Hospital Clinico Universitario/INCLIVA. Spain.

[2]Laboratory "Movement Sport and health Sciences", University Rennes. France

[3]Department of Physiology. University Complutense of Madrid. Spain

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Address correspondence to:
Jose Viña, Professor, MD (Corresponding author)
Department of Physiology. Faculty of Medicine. University of Valencia.
Av. Blasco Ibañez, 15, Valencia
46010 Spain
Phone: (34) 96 386 46 50
Fax: (34) 96 386 46 42
Email: jose.vina@uv.es

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Running title: Growth hormone as antioxidant

ABSTRACT

The aim of our study was to elucidate the role of GH replacement therapy in three of the main mechanism involved in sarcopenia: alterations in mitochondrial biogenesis, increase in oxidative stress, and alterations in protein balance.

We used young and old Wistar rats that received either placebo or low doses of GH to reach normal IGF-1 values observed in the young group.

We found an increase in lean body mass and plasma and hepatic IGF-I levels in the old animals treated with GH. We also found a lowering of age-associated oxidative damage and an induction of antioxidant enzymes in the skeletal muscle of the treated animals. GH replacement therapy resulted in an increase in the skeletal muscle protein synthesis and mitochondrial biogenesis pathways. This was paralleled by a lowering of inhibitory factors in skeletal muscle regeneration and in protein degradation.

GH replacement therapy prevents sarcopenia by acting as a double-edged sword, antioxidant and hypertrophic.

INTRODUCTION

Sarcopenia is a syndrome characterised by progressive and generalised loss of skeletal muscle mass and strength with a risk of adverse outcomes such as physical disability, poor quality of life and death (Evans 1995).This loss of muscle occurs at a rate of 3-8% per decade after the age of thirty with a higher rate of muscle loss at advanced age (Holloszy 2000). Recent estimates show that one-quarter to one-half of men and women aged 65 and older are likely sarcopenic (Janssen 2004). Progressive sarcopenia is ultimately central to the development of frailty, an increased likelihood of falls, and impairment of the ability to perform activities of daily living(Evans 1995). The logical endpoint of severe sarcopenia is loss of quality of life and ultimately institutionalization (Wolfe 2006).

The importance of maintaining muscle mass and physical and metabolic functions in the elderly is well-recognized. Less appreciated are the diverse roles of muscle throughout life and the importance of muscle in preventing some of the most common and increasingly prevalent clinical conditions, such as obesity and diabetes (Wolfe 2006). Skeletal muscle atrophy is a common feature in several chronic diseases and conditions. It reduces treatment options and positive clinical outcomes as well as compromising quality of life and increasing morbidity and mortality (Wolfe 2006). Individuals with limited reserves of muscle mass respond poorly to stress (Wolfe 2006). In support of the importance of maintaining skeletal muscle mass, strength and function, a recent study has demonstrated that all-cause, as well as cancer based, mortality, is lowest in men in the highest tertile of strength, an indicator of high muscle mass (Ruiz et al. 2008).

If there is a pre-existing deficiency of muscle mass before trauma, the acute loss of muscle mass and function may push an individual over a threshold that makes recovery of normal function unlikely to ever occur. For this reason, >50% of women older than 65 years who break a hip in a fall never walk again (Cooper 1997).

Several hormones have been suggested to have an impact on muscle mass, strength and function (Cruz-Jentoft 2012). Among them, growth hormone (GH) has been one of the most studied (Cruz-Jentoft 2012). Levels of GH are usually lower in the elderly subjects and the amplitude and frequency of pulsatile GH release are significantly reduced (Cruz-Jentoft 2012). Thus it has been hypothesized that GH would be useful in preventing the age-related loss of muscle mass (Giannoulis et al. 2012).

In our study we aimed to elucidate the role of GH replacement therapy in four of the main mechanisms involved in the onset and progression of sarcopenia: alteration in mitochondrial biogenesis, increase in oxidative stress, increase in protein degradation, and lowering in the rate of protein synthesis (Doherty 2003; Derbré et al. 2012).

In this study, we present the existing evidence behind the argument that restoration of GH profile is a good intervention to improve or preserve skeletal muscle mass in old animals.

MATERIAL AND METHODS

Animals and treatment

Ten young (aged 1 month) and twenty old (aged 22 months) male Wistar rats, maintained under controlled light and temperature conditions, were used in the study. We chose 22 month-old rats because previous studies have reported that sarcopenia is evident at this age in this species (Hopp 1993). The animals were fed a normal rat chow (A.04; Panlab, Barcelona, Spain) and had free access to tap water. Half of the old animals (n=10) were treated daily with two subcutaneous doses of GH (2mg/kg/d from Omnitrope, Sandoz, Spain, diluted in saline) one at 10.00 and another at 17.00 h for 8 weeks. Control animals were injected with the same amount of vehicle (saline solution) as GH-treated rats. After eight weeks of treatment, rats were sacrificed by cervical dislocation followed by decapitation and troncular blood was collected and processed to measure plasma IGF-I. Gastrocnemius muscle, liver, and heart were collected and immediately frozen in liquid nitrogen. The study was conducted following recommendations from the institutional animal care and use committee, according to the Guidelines for Ethical Care of Experimental Animals of the European Union. The Committee of ethics in research from the University Complutense of Madrid granted ethical approval.

We have previously shown that young animals do not show any effect when submitted to our GH treatment because they have high endogenous GH levels and also do not show alterations that could get ameliorated (Castillo et al. 2004; Carmen Castillo et al. 2005; C Castillo et al. 2005). This is why this experimental group has not been included in the study.

Body composition study

All rats were weighted weekly to determine changes in body weight during the study. After the rats were sacrificed total body fat was determined by the Specific Gravity Index (SGI), which shows the proportion between lean mass and body fat (López-Luna et al. 1986). This can be calculated comparing the animal's carcass weight (animal without head, hair and viscera) in the air (Wa) and in the water (Ww), using the following formula: SGI = Wa/[(Wa–Ww)] (assuming the specific gravity of water at 21°C to be one) (López-Luna et al. 1986).

IGF-I levels

Plasma and hepatic IGF-I levels were measured as previously described (Rol De Lama 2000) by an specific radioimmunoassay, using reagents kindly provided by the National Hormone and Pituitary Program from the National Institute of Diabetes and Digestive and Kidney Diseases and a secondary antibody obtained in our laboratory.

Determination of oxidative damage in gastrocnemius muscle

Oxidative modification of total proteins in gastrocnemius muscles was assessed by immunoblot detection of protein carbonyl groups using the "OxyBlot" protein oxidation kit (Millipore, Massachusetts) as previously described (Romagnoli et al. 2010).
Oxidative DNA damage was measured by 8-hydroxy-2′-deoxyguanosine (8-OHdG). A commercially available enzyme linked immunoassay (Highly Sensitive 8-OHdG Check, Japan Institute for the Control of Aging, Japan) was used to measure oxidized DNA in isolated muscle DNA samples. DNA was extracted from the muscle via the High Pure PCR Template Preparation Kit (Roche, GmbH, Germany) according to the manufacturer's protocol. DNA was used if it had a minimum 260:280 ratio of 1.8. The assay was performed following the manufacturer's directions. Briefly, 50 µl of DNA were incubated with the primary antibody, washed, and then incubated in secondary antibody. The chromogen (3,3′,5,5′-tetramethylbenzidine) was added to each well, and incubated at room temperature in the dark for 15 min. The reaction was terminated and the samples were read at an absorbance of 450 nm. Samples were normalized to the DNA concentration measured via a plate spectrophotometer for nucleic acids (ND-2000, NanoDrop, Wilmington, DE). All analyses were done in triplicate.

Determination of citrate synthase and glucose-6-phosphate dehydrogenase (G6PDH) activities in gastrocnemius muscle

Citrate synthase assay was performed in the gastrocnemius muscle following the method of Srere (Srere 1969). Results were obtained in nmol x mg of protein^{-1} x min^{-1}. Values were normalized to those observed in the samples obtained from the young group, which were assigned a value of 100%.
Glucose-6-phosphate dehydrogenase activity was determined following the method of Waller and co-workers(WALLER et al. 1957). Results have been expressed in nmol x mg of protein^{-1} x min^{-1}.

Protein concentrations were determined by Bradford's method (Bradford 1976) by using bovine serum albumin as standard.

Immunoblot analysis

Aliquots of muscle lysate (50-120 µg of proteins) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The whole gastrocnemius was used to ensure homogeneity. Proteins were then transferred to nitrocellulose membranes, which were incubated overnight at 4 °C with appropriate primary antibodies: anti-myf5 (1:200, Santa Cruz Biotechnology Inc, Santa Cruz, CA), anti-p70S6K (1:1000, Cell Signaling); anti-phosphorylated p70S6K (1:1000, Cell Signaling); anti-myostatin (1:1000, Abcam, UK); anti-catalase (1:5000, Sigma Aldrich, Missouri); anti-G6PDH (1:1000, Abcam, UK); anti-Gpx (1:2000, Abcam, UK); anti-cytochrome C (1:1,000, Santa Cruz Biotechnology, CA), anti-PGC-1α (1:1000, Cayman); anti-AKT (1:1000, Cell Signaling); anti-phosphorylated AKT (1:1000, Cell Signaling); anti-p38 (1:1000, Cell Signaling); anti-phosphorylated p38 (1:1000, Cell Signaling); anti-MuRF1 (1:200, Santa Cruz Biotechnology Inc, Santa Cruz, CA); anti-MAFbx (1:500, Abcam, UK); anti-Nrf1 (1:200, Santa Cruz Biotechnology Inc, Santa Cruz, CA); and anti-p21 (1:200, Santa Cruz Biotechnology Inc, Santa Cruz, CA). Thereafter, membranes were incubated with a secondary antibody for 1 h at room temperature. Specific proteins were visualized by using the enhanced chemiluminescence procedure as specified by the manufacturer (Amersham Biosciences, Piscataway, NJ). Autoradiographic signals were assessed by using a scanning densitometer (BioRad, Hercules, CA). Data were represented as arbitrary units of immunostaining. To check for differences in loading and transfer efficiency across membranes, an antibody directed against α-actin (1:1000, Sigma Aldrich Missouri) was used to hybridize with all the membranes previously incubated with the respective antibodies. For the Western Blotting quantifications we first normalized all the proteins measured to α-actin. Samples from each group were run on the same gel.

Statistical Analysis

Statistical analyses were performed using the SigmaStat 3.1 Program (Jandel Corp., San Rafael, CA). Results are expressed as mean ± SD. Normality of distribution was checked with the Kolmogorov test and homogeneity of variance was tested by Levene's statistics. We used one-way ANOVA to compare group differences. If overall ANOVA revealed significant differences, post hoc (pairwise) comparisons were performed using Tukey's test. Differences were considered significant if $p<0.05$.

RESULTS

Effect of ageing and GH replacement therapy on body composition of rats

Table 1 shows the effect of ageing on body composition of rats. In the two months study period young animals increased their weight by 20 g (6.9 % of their initial weight) whereas old animals lost weight by approximately 60 g (-9.8% of their initial weight). However, when old animals were treated with GH they showed an increase in weight of approximately 9 g (1.5% of their initial weight), i.e., very significantly different from the loss of weight that occurred in old untreated rats. This loss in weight was mainly due to changes in lean mass because the SGI fell from 5 in young animals to 3 in old ones, which mean that adiposity is augmented and lean body mass reduced. Old treated animals had an intermediate SGI, i.e., 4. We measured the gastrocnemius atrophy by weighting the muscle and we found a significant (30%) decrease in the relative muscle weight in the old animals, that was significantly prevented in the old treated ones. It is well-known that GH increases the weight of the heart. We show that the relative weight of the heart of old animals fell in the study period and that treatment with GH resulted in a significant increase in the relative heart weight (see Table 1).

IGF-1 levels are known to fall with age and this is what we report in Table 1. Young animals had plasma IGF-1 levels of approximately 1100 ng/mL and this fell to 600 ng/mL in old animals. Old animals treated with GH showed a very significant increase in IGF-1 that went from approximately 600 (in old untreated) to approximately 1200 ng/mL in old animals treated with GH, i.e., an increase of 100%. We also determined the hepatic IGF-1 levels and similar results were found, old animals showed a decrease in their liver IGF-1 levels that was prevented by treatment with GH.

GH replacement therapy prevents age-associated oxidative damage to skeletal muscle

Figure 1 reports the effect of ageing on protein and DNA oxidation and its prevention by GH replacement therapy. Panel A shows results of the effect of ageing on skeletal muscle protein oxidation. Ageing resulted in an increase in protein oxidation that was relatively small (approximately 10%) but statistically significant. This was prevented by treatment with GH, thus old animals treated with GH had values of protein oxidation that were not distinguishable from those of young animals. In Panel B we report results on DNA oxidation. Old animals

had a significantly increased DNA oxidation (as determined by the levels of 8-OHdG) when compared with young controls. Treatment with GH completely prevented this increase.
Thus, protein as well as DNA oxidation are elevated in muscles of old animals but this is prevented by treatment of old animals with GH.

Effect of ageing and GH replacement therapy on antioxidant enzyme levels in skeletal muscle of rats

To seek an explanation for the effect of GH replacement therapy in protecting against oxidative damage, we measured the levels of three important antioxidant enzymes, catalase, glutathione peroxidase, and G6PDH, the latter being an antioxidant because it generates NADPH required for normal functioning of the glutathione redox cycle, and because it activates catalase (M. D. Scott et al. 1993). Treatment with GH increased the levels of antioxidant enzymes (see Figure 2). The effect of ageing itself on the levels of these enzymes was marked and significant from the statistical viewpoint in the case of G6PDH. We also determined the G6PDH enzymatic activity and we confirmed our Western Blotting's results. Young animals had a skeletal muscle G6PDH activity of 0.49 ± 0.09 nmol x mg of protein^{-1} x min^{-1}. We found a significant ($p<0.01$) decrease in its activity in the old animals (0.28 ± 0.04 nmol x mg of protein^{-1} x min^{-1}) that was recovered in the old group treated with GH (0.44 ± 0.08 nmol x mg of protein^{-1} x min^{-1}) ($p<0.01$). However, we did not find a significant effect of ageing on catalase and glutathione peroxidase protein levels. In any case, there was a clear up-regulation of these enzymes when we treated old animals with GH. These effects explain the prevention of age-associated damage to muscle proteins and DNA by GH and indeed suggest a so-far unknown antioxidant effect of GH.

Effect of ageing and GH replacement therapy on mitochondriogenesis in rat muscle

We have previously observed that mitochondriogenesis (which is heavily dependent on the activity of PGC-1α) is seriously affected by oxidative stress (Derbré et al. 2012). The observation that GH prevents age-associated oxidative stress in muscle, prompted us to test whether PGC-1α was affected in old animals and whether treatment with GH could reverse this effect. Figure 3 shows that PGC-1α levels were significantly lower in old animals than in young ones. This decrement (see Panel A) was completely prevented by replacement therapy with GH. Similar results were found with the protein levels of NRF-1. PGC-1α co-activates

NRF-1 and as expected we found a significant decrease in the NRF-1 levels in the old animals that was prevented by treatment with GH (see Figure 3 Panel B). Since PGC-1α is the master regulator of mitochondriogenesis, we tested whether GH replacement therapy in old animals resulted in changes in mitochondrial mass and for this we used two markers, cytochrome C protein levels and citrate synthase activity. Figure 3 (Panels C and D) shows that levels of either cytochrome C or citrate synthase activity were significantly lower in old animals than in young ones and that this decrease was fully prevented when old animals were treated with GH. So we can conclude that mitochondriogenesis in old animals, which depends on PGC-1α, is seriously depressed in old animals (as already well established) but that this is prevented by treatment with replacing doses of GH.

Effect of ageing and GH replacement therapy on skeletal muscle protein synthesis

It is well established that skeletal muscle mass is lower in old animals than in young ones and that GH may have an effect in preventing this (C Castillo et al. 2005; Carmen Castillo et al. 2005; Castillo et al. 2004). However, so far it is not clear whether the effect of GH on muscle mass is due to suppression in protein degradation, increase in amino acid uptake and/or stimulation of protein synthesis. The insulin family ligands can bind to the IGF-I receptor which then phosphorylates IRS-1(Clemmons 2009). This protein acts as a docking protein for activation of PI-3 Kinase(Clemmons 2009). PI-3K activation leads to phospholipid generation in the plasma membrane, which recruit and activate AKT Kinase. We found a significant decrease in the phosphorylation of AKT in the old muscles that was completely recovered when the old animals were treated with GH (See Figure 4 Panel A). AKT Kinase activation leads to activation of mTOR and of the mitogen-activated serine/threonine kinase p70 ribosomal protein S6 kinase (p70S6K). Recent work in humans has identified the mammalian target of rapamycin complex I (mTORC1) as being required to stimulate muscle protein synthesis in humans(Dickinson et al. 2011). p70S6K, a downstream target of mTORC1, plays a critical role in cell growth and survival (Baar & Esser 1999; Bodine et al. 2001; Dorn & Force 2005; Wu et al. 2009). We have found that the phosphorylation of p70S6K decreases with ageing and is restored to young values with GH replacement therapy (see Figure 4 Panel B).

Myf-5 is a primary myogenic regulatory factor. It facilitates repair or regeneration and growth of mature myofibers. Figure 4 (Panel C) reports results on myf-5 protein levels in young and old animals and the effect of GH replacement therapy. Although ageing did not

affect the basal muscle levels of myf-5, we found a significant increase in this myogenic factor in the GH treated group.

Effect of ageing and GH replacement therapy on the expression of inhibitory growth factors in skeletal muscle

Myostatin is one of the major inhibitory factors in skeletal muscle regeneration. It down-regulates myf-5 and myoD. We found a significant increase in myostatin in the old skeletal muscle that was prevented with GH replacement therapy (Figure 5 Panel A). Myostatin maintains satellite cell quiescence and repress cell-renewal through the induction of p21 (McCroskery et al. 2003), which is a cell cycle inhibitor (Jaumot et al. 1997). p21 expression is significantly (40%) increased in old animals when compared to young ones, this is prevented by treatment with GH (see Figure 5 Panel B). Recently we have shown that p38 signaling promotes skeletal muscle atrophy through the expression of E3 ubiquitin ligases(Derbre et al. 2012). Figure 5 (Panel C) shows an increase in the phosporylation of p38 MAPKinase in the muscle of old animals. GH treatment significantly prevented the p38 phosphorylation. To finally identify the mechanism by which GH prevents the loss of muscle mass during ageing we determined the expression, in gastrocnemius muscle, of the Muscle atrophy F-Box (MAFbx) and Muscle RING Finger-1 (MuRF1). MAFbx and MuRF1 are two well known muscle specific E3 ubiquitin ligases involved in proteolysis. We did not find a significant effect of ageing on MAFbx protein levels (see Figure 5 Panel E). However, aging was associated with a significant increase in MuRF1 that was prevented by treatment with GH.

Collectively the data reported in figures 4 and 5 show that the combined effect of the up regulation of myostatin, p21, p38 and MuRF1, and the down regulation of AKT-p70S6K and myf-5 may be involved in the lowering in protein levels in the skeletal muscle of old rats. GH replacement therapy seems to be a good strategy to restore skeletal muscle regeneration and to combat the process leading to sarcopenia.

DISCUSSION

Effect of ageing and GH replacement therapy on body composition of rats

There are three general approaches to hormone therapy. Hormones can be given to replace a deficiency, to raise their concentration above the normal value, and finally agents can be given to block hormone action by either reducing the rate of secretion or by blocking their action (Wolfe 2006). Despite the large number of studies aiming at assessing the effects of GH supplementation on muscle mass, the controversial findings reported in the literature, maintain the debate as to whether or not to use GH to treat sarcopenia (von Haehling et al. 2012). The contrasting findings reported may be explained by methodological differences such as dosing. High doses of GH cause high incidence of adverse effects (Papadakis et al. 1996; Holloway et al. 1994). Thus, we have used relatively low doses of GH in our study. We need to take into consideration that the GH used was of human origin, so that the response was not the same as for rat GH. On the other hand, small animals need a much higher dosage than humans, as was demonstrated by Mordenti and co-workers (Mordenti et al. 1991). Plasma IGF-1 values were lower in old than in young animals but IGF-1 levels in old animals treated with GH were not statistically different from young controls. We also determined the hepatic IGF-1 levels and found similar results, an age-associated decrease in the liver IGF-1 levels that was prevented by the treatment with GH (See Table 1).

In our hands, GH replacement therapy is useful in preventing the age-related muscle mass loss. In the two month study period we found that young animals increased their weight whereas old animals lost weight (see Table 1). However, when old animals were treated with GH they showed an increase in weight of approximately 9 g, i.e., significantly different from the loss of weight that occurred in old untreated rats. This loss was mainly due to changes in lean mass because the SGI fell from 5 in young animals to 3 in old ones. SGI is an index that relates lean body mass and fat mass; the higher it is, the less fat the animal has. Our data also show that GH administration significantly increases SGI in old male rats, which means that GH, through its anabolic, antilipogenic and lipolytic properties, is able to increase muscle mass and reduce body fat (Castillo et al. 2004; Carmen Castillo et al. 2005). We also determined the gastrocnemius muscle atrophy by weighting the muscles, and we found a 30% decrease in the relative muscle weight in the old animals, that was significantly prevented in the old treated ones. Finally, we also found that the relative weight of the heart of old animals fell along the study period and that treatment with GH resulted in an increase in heart weight (see Table 1).

The antioxidant effect of GH replacement therapy

The free radical theory of ageing has provided a theoretical background to devise experiments to understand ageing (Gomez-Cabrera, Sanchis-Gomar, et al. 2012). It is now well established that up-regulating the endogenous antioxidant defenses is a useful mechanism for cells to prevent damage associated with excessive free radical production (Gomez-Cabrera, Domenech & Viña 2008; Gomez-Cabrera, Domenech, Romagnoli, et al. 2008). The effects of GH on sarcopenia have been studied extensively (Brill 2002; Papadakis et al. 1996), but so far they have been completely dissociated with prevention of free radical damage. A major finding reported in this paper is that GH supplementation can act as an antioxidant because it up-regulates the expression of important intracellular antioxidant enzymes, such as catalase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase (see Figure 2). The result of this up-regulation is that, as reported in Figure 1, old animals treated with relatively small doses of GH suffer less oxidative stress than untreated old animals, both in terms of protein oxidation (measured as carbonylation) and DNA oxidation (measured by the levels of 8-OHdG). Our results show that supplementation with GH activates endogenous antioxidant enzymes, prevents oxidative damage to critical cellular structures, and thus behaves as an antioxidant. This may contribute to explain the protection against sarcopenia conferred by supplementation with GH as discussed in the following paragraphs. The mechanism by which GH activated the expression of antioxidant enzymes is beyond the scope of this paper and is being studied in this laboratory.

Protein synthesis, mitochondriogenesis, and the prevention of sarcopenia by GH

The maintenance of skeletal muscle mass is regulated by a balance between protein synthesis and protein degradation (Powers et al. 2011). Muscle protein synthesis decreases with age (Jones et al. 2009). The involvement of p70S6K in skeletal muscle hypertrophy has been documented in various animal models (Y. Song et al. 2005) . When activated via AKT Kinase, mTOR influences translation initiation by involving phosphorylation of p70S6K, which, in turn, phosphorylates the S6 ribosomal protein and allows the up-regulation of a subclass of mRNAs encoding the translational apparatus (Kimball et al. 2002). As shown in Figure 4, we found a significant decrease in the phosphorylation of AKT in the skeletal muscle of the old animals that was completely recovered when they were treated with GH. Similarly, phosphorylation of p70S6K was lower in old skeletal muscles than in young ones and this was not caused by changes in total p70S6K protein levels. Old animals treated with GH showed similar phospho-p70S6K values than young animals (See Figure 4). Our results

contradict previous studies showing that an intraperitoneal injection of IGF-I increases phosphorylation of p70S6K in the young but not in the old skeletal muscle (M. Li et al. 2003).

The attenuation in the capacity for muscle hypertrophy in old individuals has also been related to an age-related impairment in myogenic potential (Marsh & Criswell 1997; Hansen et al. 2007). Thus, we aimed to compare the myogenic response of gastrocnemius muscle in young and old rats treated with GH. Myf-5 is a well-known marker of myoblast/satellite cell differentiation and facilitates repair or regeneration and growth of mature myofibers (Kim et al. 2005). It has been shown that GH treatment up-regulates, not only liver IGF-I, but also skeletal muscle IGF-I gene expression (Hameed et al. 2003) that is involved in the activation of satellite cells (Goldspink & Harridge 2004). Figure 4 shows that although ageing did not cause a decrease in the myf-5 skeletal muscle protein levels, GH replacement therapy significantly increased the levels of this myogenic factor.

We then focused our interest in myostatin, a negative muscle regulatory factor (Goldspink & Harridge 2004). This belongs to the TGFβ family, but its expression is restricted to muscle tissue (McPherron & Lee 1997). Absence or blockade of myostatin induces massive skeletal muscle hypertrophy that was initially attributed to the proliferation of the population of muscle fiber-associated satellite cells (Ten Broek et al. 2010). However, it has been recently shown that myostatin regulates protein balance within the muscle fibers themselves. Several research groups have shown that hypertrophy, in the absence of myostatin, involves little or no input from satellite cells (Amthor et al. 2009; Welle et al. 2006). Hypertrophic fibers contain no more myonuclei or satellite cells and myostatin has no significant effect on satellite cell proliferation *in vitro* (Amthor et al. 2009). As previously reported, we found an increase in myostatin and p21 in old muscles (McKay et al. 2012). GH replacement therapy significantly reduced them (see Figure 5). Thus, the effect of GH on these two factors may contribute to the prevention of muscle wasting. Phosphorylation of p38-MAPK has been reported after addition of myostatin in skeletal muscle fibroblasts (Li et al. 2008). p38 is a stress-activated protein kinase that responds to a variety of stimuli, including oxidative stress and TNF-α (Derbre et al. 2012), and has been identified as a likely mediator of catabolic signaling in skeletal muscle (Powers et al. 2007; Li et al. 2005). Thus, we determined the phosphorylation of p38 MAPK in the gastrocnemius muscle samples. As previously reported (Williamson et al. 2003)we found a significant increase in p-p38 in the old animals that was prevented by hormone replacement therapy with low doses of GH.

To identify the final mechanism by which GH prevents the loss of muscle mass during aging we determined the expression of two well known muscle specific E3 ubiquitin ligases involved in several *in vivo* models of skeletal muscle atrophy, MAFbx and MuRF1(Foletta et al. 2011). Although controversial (Edström et al. 2006), the levels of both MuRF1 and MAFbx mRNA are markedly up-regulated in aged muscles (Clavel et al. 2006). We found a significant increase in muscle MuRF1 protein levels in the old animals that was prevented by GH treatment. However, we did not find any change on muscle MAFbx protein levels. Thus, MuRF1 seems to be involved in the age-associated sarcopenia.

Ageing causes a decrease in mitochondrial content and activity (Miquel et al. 1980; Sastre et al. 1996). PGC-1α is a master regulator of mitochondrial biogenesis (Puigserver et al. 1998; Viña et al. 2009) and itself is a molecule that responds swiftly to the changes in oxidative stress(St-Pierre et al. 2006; Viña et al. 2009; Gomez-Cabrera, Domenech & Viña 2008). Since, as described in the previous paragraph, we have seen that ageing in muscle results in an increase in oxidative stress markers and that this is prevented by relatively low doses of GH, we tested whether ageing resulted in a decrease in PGC-1α expression in muscle and this was indeed the case, as shown in Figure 3. Treatment with GH completely prevents the decrease in PGC-1α associated with ageing. PGC-1α co-activates NRF-1 and we found that the levels of NRF-1 were significantly lower in the skeletal muscle. This was completely prevented when animals were treated with GH. We previously reported that PGC-1α does not respond to the normal activation by exercise when animals are old (Derbré et al. 2012). This lack of responsiveness may be due to a blocking of the activating mechanisms by GH because when it is administered to animals, PGC-1α is activated and mitochondriogenesis resumes, as shown in Figure 3. Probably GH activates PGC-1α, acting in three ways. Stimulating the IGF→AKT→ mTOR→ p70S6K pathway, inhibiting the Myostatin→p38→ MuRF1, and acting as an antioxidant (as described above). Recently Vescovo et al., working in cardiac muscle, reported that GH activates PGC-1α via IGF-1 and calcineurin (Vescovo et al. 2005). Our previous work showing that PGC-1α could also be activated by MAP kinases together with the antioxidant effects discussed in the previous paragraph, may explain the unique capability of GH supplementation to maintain normal muscle levels in the old animal. Our previous work (Derbré et al. 2012) showed that neither exercise, cold exposure, or even thyroid hormone treatment could activate PGC-1α in the old animal. A marker of mitochondrial mass, cytochrome C protein levels, were also lower in the muscle from old animals. We also found that the citrate synthase activity was 50% lower in the muscles of our old animals than in the young ones. It has been reported that mitochondrial isolation

procedures induce preferential losses of matrix soluble enzymes, such as citrate synthase, in aged muscle mitochondria(Johnson et al. 2013). In a recent study, Picard and co-workers examined the differences in mitochondrial function between permeabilized muscle fibers and isolated mitochondria from the same sample (Picard et al. 2010). The authors found that mitochondrial function was decreased in both isolated mitochondria and permeabilized fibers, with an exaggerated age-effect in isolated mitochondria (Picard et al. 2010). We cannot rule out the idea that the dramatic decrement found in the citrate synthase activity in our study migh have been due to sample preparation. However, we consider that collectively the results reported in Figure 3 supports the idea that GH replacement therapy prevents the age associated decline in mitochondrial content in the old skeletal muscle.

In this respect, the activation by GH that we report here seems to be unique in maintaining normal muscle mass in the old animal and thus preventing sarcopenia.

In this study, we report results supporting the argument that restoration of GH profile is a good intervention to preserve skeletal muscle mass in the elderly. A schematic interpretation of our results is in Figure 6. We would like to reiterate that the supplementation of GH that we have performed in the animals is a rather low one in that the aim is to return the levels to the normal physiological ones. We do not claim here that supplementation with high doses of GH should be recommended. However, small doses of GH supplementation may be a very useful way to prevent age-associated sarcopenia. If these results could be extrapolated to humans, one could suggest that the loosing of muscle mass observed in persons, even if they have performed exercise in their youth, could be prevented by hormone replacement therapy with low doses of GH. This interesting possibility remains to be studied in the clinical setting.

ACKNOWLEDGEMENTS

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TABLE

Table 1. Effect of ageing and GH replacement therapy on body composition and IGF-1 plasma and hepatic levels in rats.

The values are shown as mean ± SD. Differences were checked for statistical significance by a one-way ANOVA. $^{a}p<0.05$, $^{aa}p<0.01$ young *vs* old; $^{b}p<0.05$, $^{bb}p<0.01$ old *vs* old treated with GH; $^{cc}p<0.01$ young *vs* old treated with GH.

SGI stands for Specific Gravity Index.

	Young (n=10)	Old (n=10)	Old + GH (n=10)
Total Final body weight (g)	311.2±14.1	569.5±75.0^{aa}	616.7±26.6^{cc}
Weight change during the two months study period (g)	+19.9±4.4	-61.9±36.2^{aa,bb}	+9.3±5.1
Weight change during the two months study period (% of initial weight)	+6.9±4.8	-9.8±5.7^{aa,bb}	+1.5±0.01
Relative gastrocnemius muscle weight (g/100g body weight)	0.52±0.03	0.36±0.02^{aa}	0.41±0.01^{b,cc}
SGI	5.0±0.1	3.0±0.6^{aa}	4.0±0.3^{bb}
Relative cardiac weight (g/100g of body weight)	0.26±0.02	0.21±0.00^{a}	0.25±0.01^{b}
Plasma IGF-1 levels (ng/mL)	1103±61	590±70^{a}	1180±90^{bb}
Hepatic IGF-1 levels (ng/mL)	237±8	192±19	324±51^{b}

FIGURES

Figure 1. GH replacement therapy prevents age-associated oxidative damage in skeletal muscle of rats.

Thirty animals were divided into three experimental groups: Young (Y) (n=10), Old (O) (n=10), and Old treated with GH (OGH) (n=10). Panel A shows a Western blotting analysis to detect protein carbonylation in gastrocnemius muscle. A representative blot is shown. For the densitometric analysis of the results, values are shown as mean (±SD). Panel B shows 8-OHdG from DNA extracted from gastrocnemius muscle of rats (Panel B). Values were normalized to those observed in the samples obtained from the young group, which was assigned a value of 100%. *p<0.05, NS: Not significantly different.

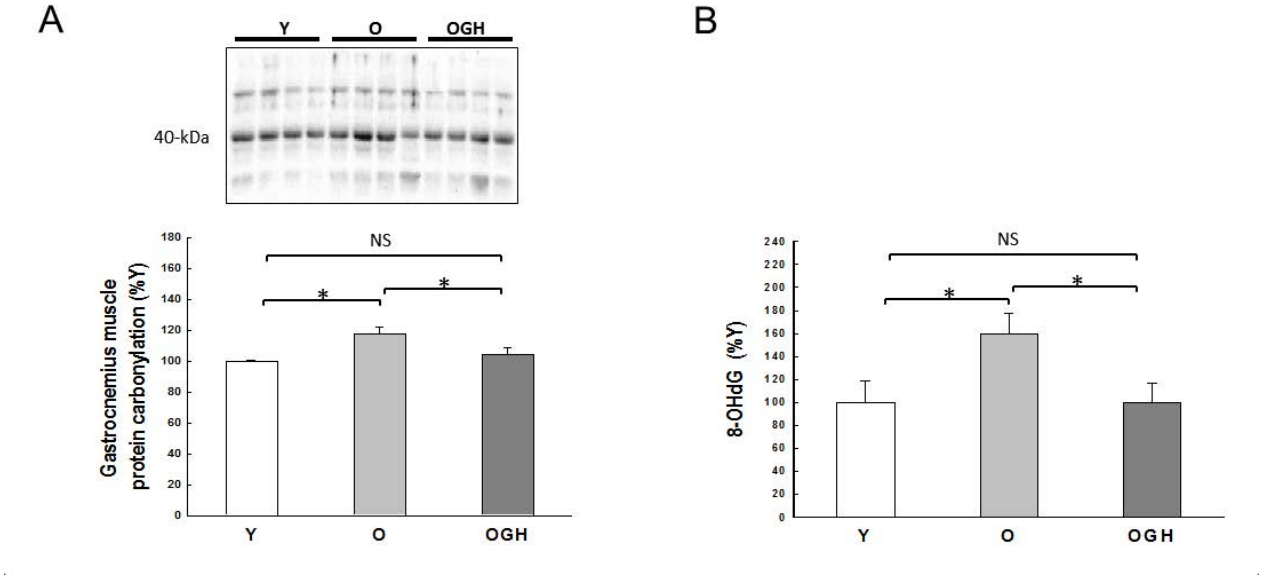


Figure 2. GH replacement therapy restores the age-associated decrease in the protein levels of antioxidant enzymes in rat skeletal muscle.

Thirty animals were divided into three experimental groups: Young (Y) (n=10), Old (O) (n=10), and Old treated with GH (OGH) (n=10). Western blotting analysis to detect catalase (Panel A), glutathione peroxidase (Panel B), and Glucose-6-phosphate dehydrogenase (Panel C) in rat gastrocnemius muscle were performed. Representative blots are shown. The content of α-actin, a housekeeping protein marker in skeletal muscle, was determined in all the experimental groups. For the densitometric analysis of the results, values are shown as mean (±SD). Values were normalized to those observed in the samples obtained from the young group, which was assigned a value of 100%. ** $p<0.01$, NS: Not significantly different.

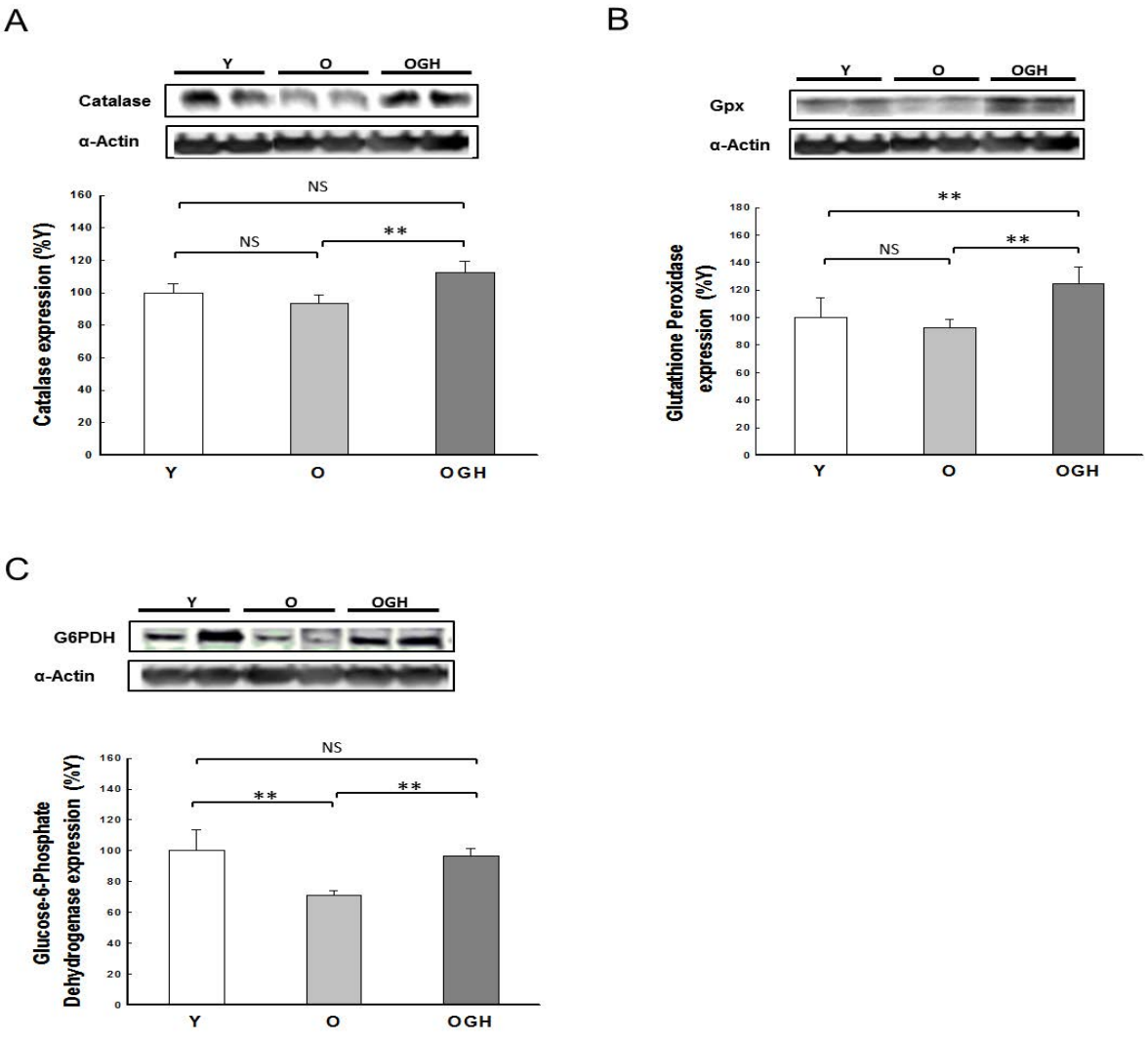


Figure 3. GH replacement therapy prevents the age-associated impairments in the skeletal muscle mitochondrial content.

Thirty animals were divided into three experimental groups: Young (Y) (n=10), Old (O) (n=10), and Old treated with GH (OGH) (n=10). Western blotting analysis to detect PGC-1α (Panel A), Nrf-1 (Panel B), and Cytochrome C (Panel C) in rat gastrocnemius muscle were performed. Representative blots are shown. The content of α-actin, a housekeeping protein marker in skeletal muscle, was determined in all the experimental groups. For the densitometric analysis of the results, values are shown as mean (±SD). Values were normalized to those observed in the samples obtained from the young group, which was assigned a value of 100%. Panel D shows citrate synthase enzymatic activity. Values were normalized to those found in the samples from the young group which was assigned a value of 100%. *p<0.05, **p<0.01, NS: Not significantly different.

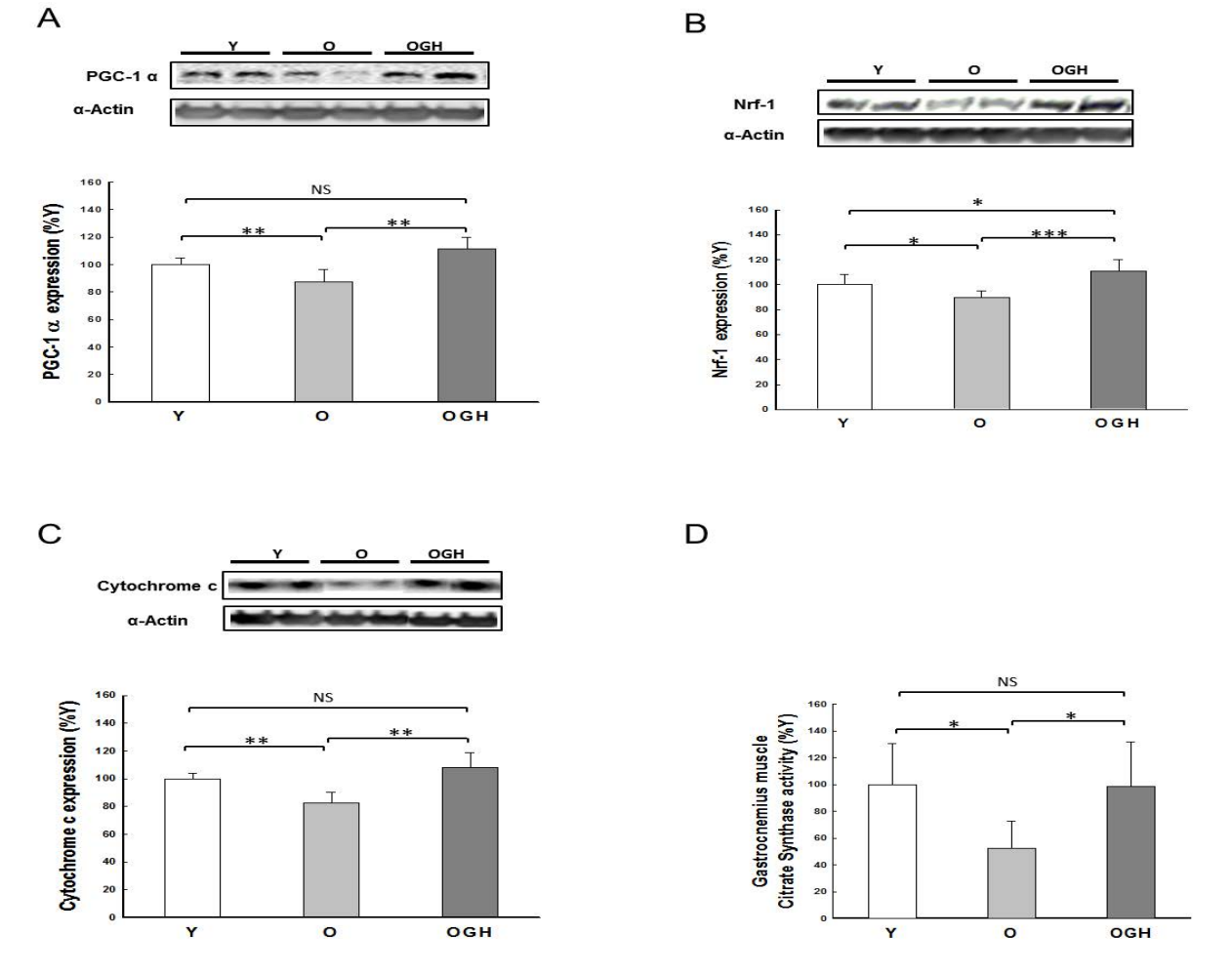


Figure 4. GH replacement therapy activates protein synthesis in the old skeletal muscle. Thirty animals were divided into three experimental groups: Young (Y) (n=10), Old (O) (n=10), and Old treated with GH (OGH) (n=10). Western blotting analysis to detect AKT activation (Panel A), p70S6K activation (Panel B), and Myf-5 (Panel C) in rat gastrocnemius muscle were performed. Representative blots are shown. The content of α-actin, a housekeeping protein marker in skeletal muscle, was determined in all the experimental groups. For the densitometric analysis of the results, values are shown as mean (±SD). Values were normalized to those observed in the samples obtained from the young group, which was assigned a value of 100%. In panels A and B the data are represented as a percentage of immunostaining values obtained for the phosphorylated form of the Kinase relative to the total form. * $p<0.05$, ** $p<0.01$, NS: Not significantly different.

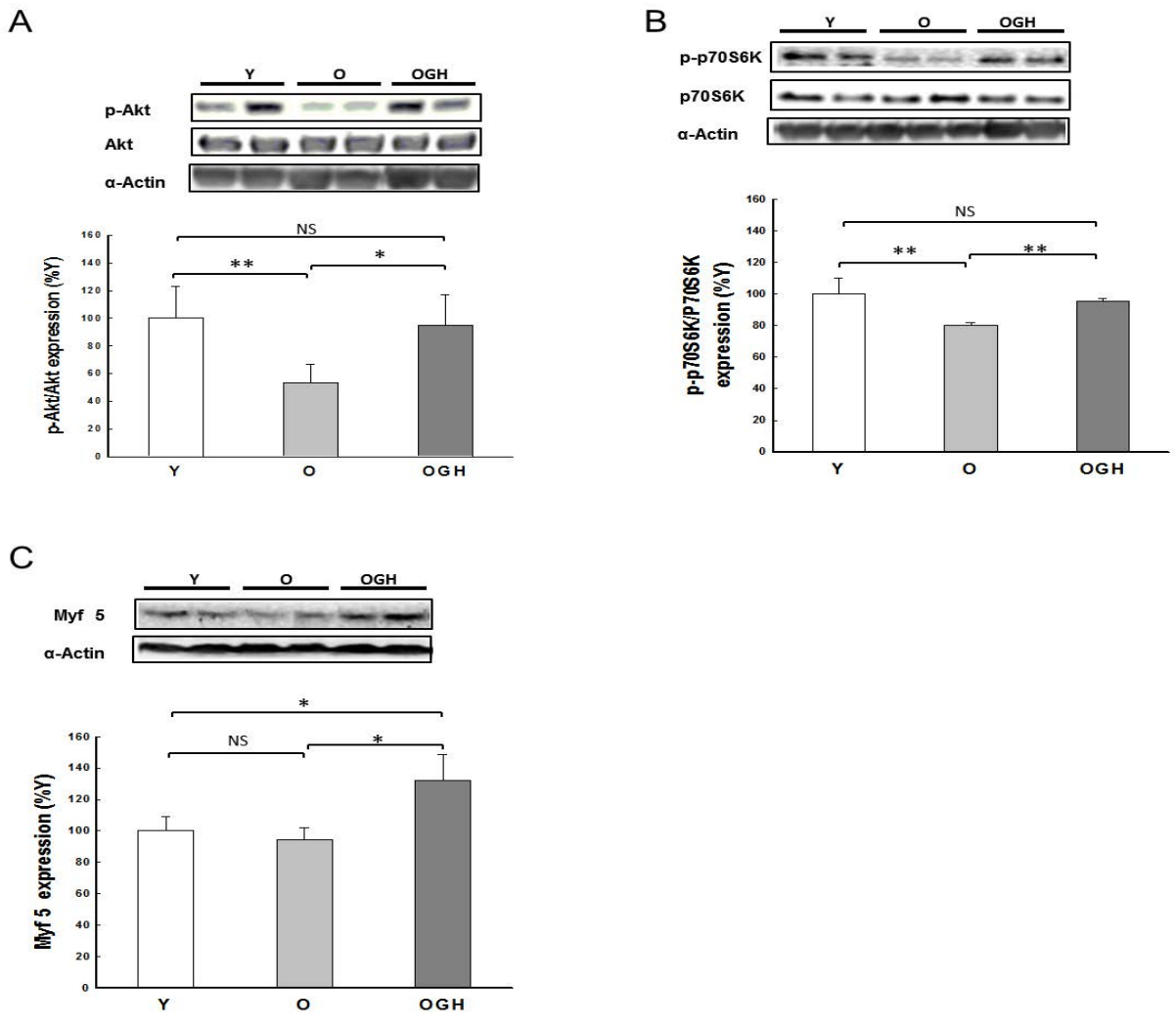
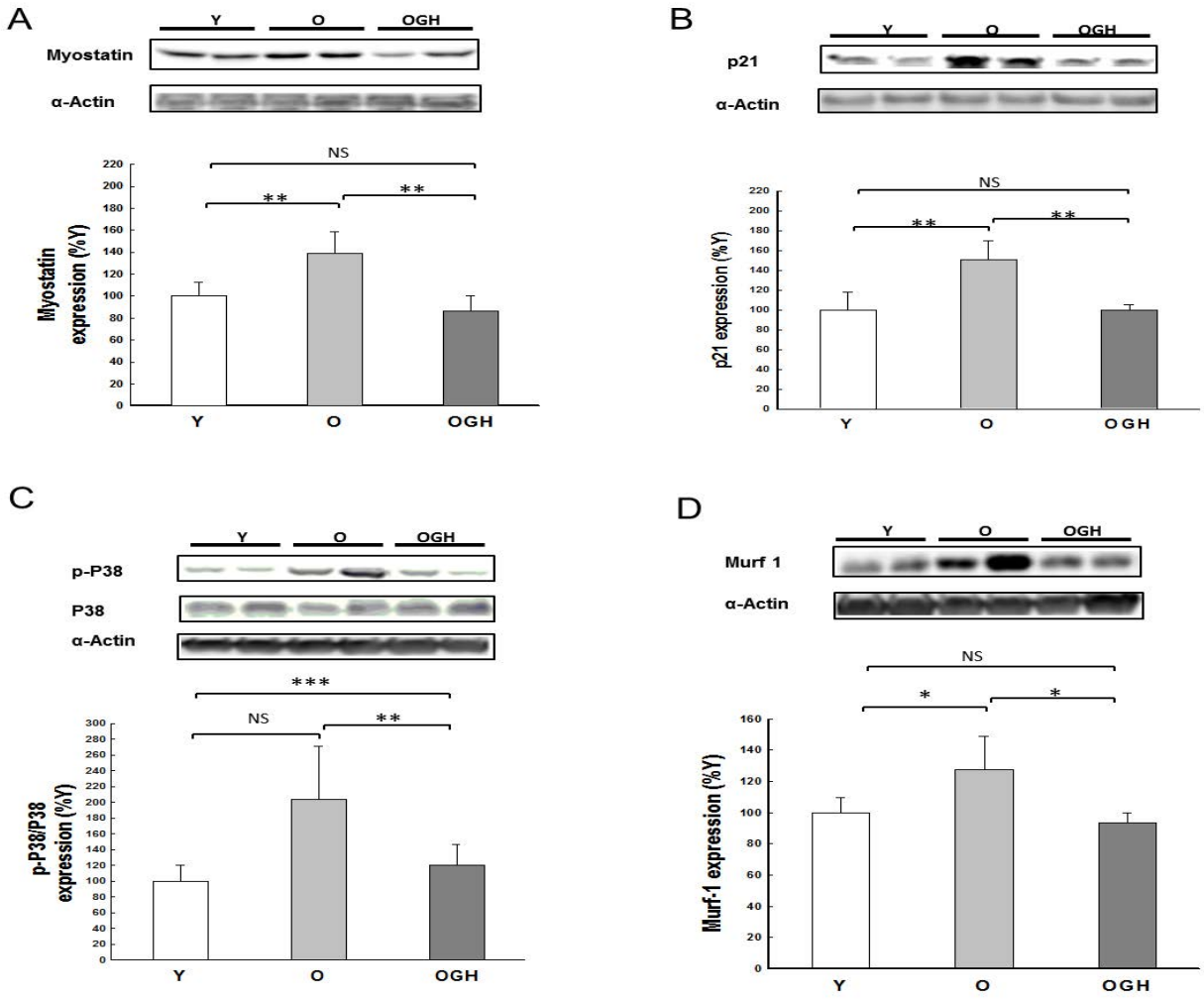


Figure 5. GH replacement therapy attenuates the age-associated increase in protein degradation in skeletal muscle.

Thirty animals were divided into three experimental groups: Young (Y) (n=10), Old (O) (n=10), and Old treated with GH (OGH) (n=10).Western blotting analysis to detect myostatin (Panel A), p21 (Panel B), P38 (Panel C), MuRF1 (Panel D), and MAFbx (Panel E) in rat gastrocnemius muscle were performed. Representative blots are shown. The content of α-actin, a housekeeping protein marker in skeletal muscle, was determined in all the experimental groups. For the densitometric analysis of the results, values are shown as mean (±SD). Values were normalized to those observed in the samples obtained from the young group, which was assigned a value of 100%. %. In panel C the data are represented as a percentage of immunostaining values obtained for the phosphorylated form of the kinase relative to the total form. *p<0.05, ** p<0.01, NS: Not significantly different.



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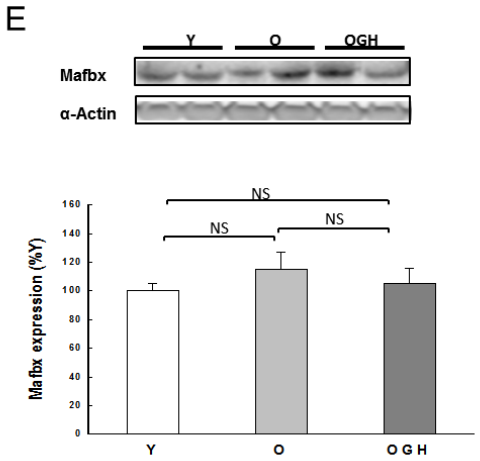
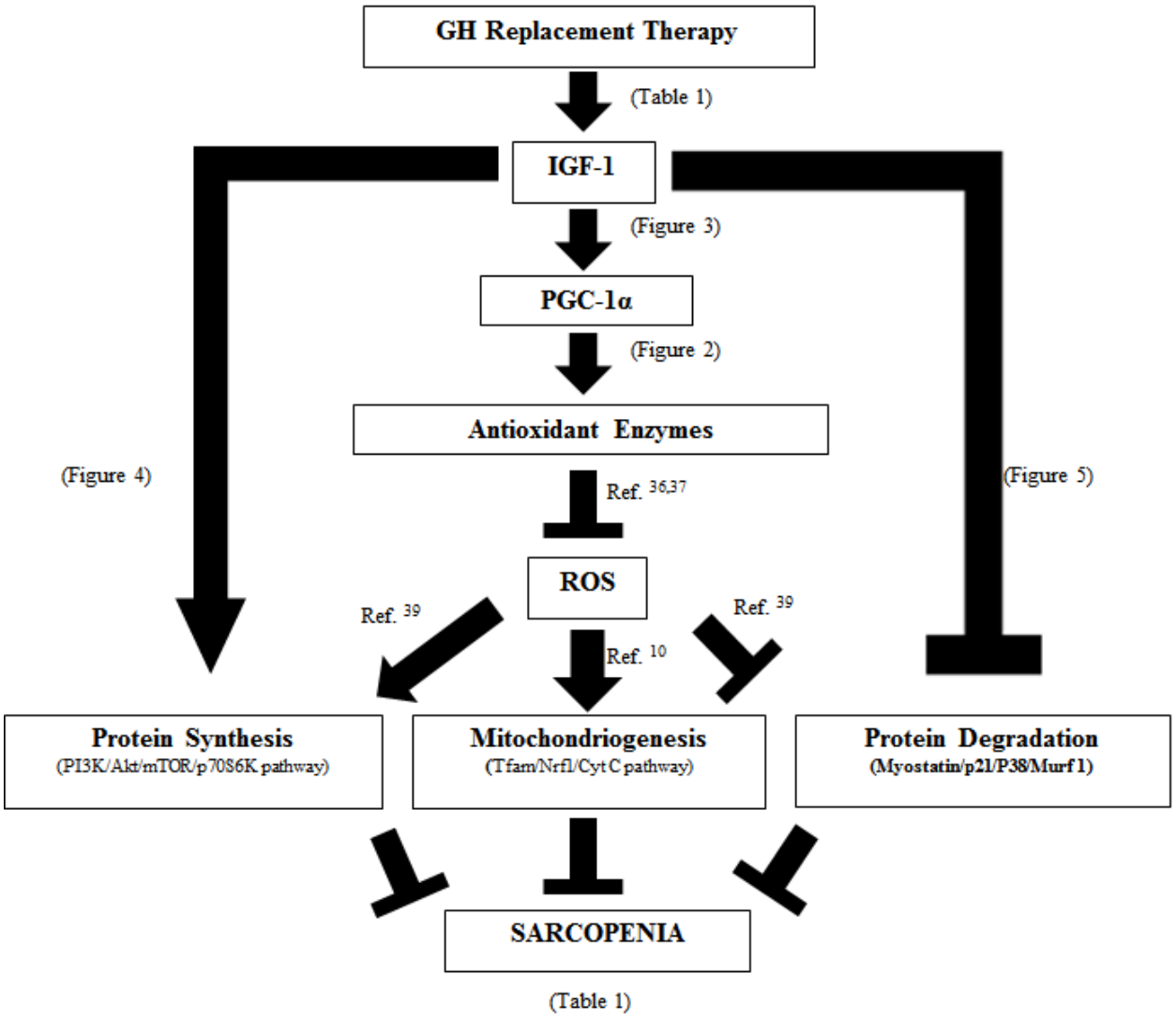

Y
O
OGH
Mafbx
α-Actin
NS
NS
NS
Mafbx expression (%Y)
160
140
120
100
80
60
40
20
0
Y
O
O G H

Figure 6. GH replacement therapy and its effects in sarcopenia.



Study 2: Glucose-6-phosphate dehydrogenase overexpression improves body composition and physical performance in mice

MATERIAL AND METHODS

Animals

Generation of a human G6PDH-transgenic (G6PDHtg) mouse model

In order to determine if a higher expression of G6PDH is effective in improving muscle mass, strength, physical performance and therefore improving healthspan in a vertebrate model, we decided to generate transgenic mice with a moderate overexpression of G6PDH under the control of its natural promoter (Figure 1). 1) For the generation of the G6PDH transgenic mice, a plasmid containing the human G6PDH (hG6PDH) entire genomic sequence (20,105 kilobases), including the entire upstream and downstream regulatory sequences, was used (Corcoran et al. 1996). 2) For transgenesis, the hG6PDH sequence was isolated from the pBluescript vector by NotI digestion and a 0,5 to 1 ng/ul DNA solution was injected into the pronuclei of F1 hybrids (C57BL/6J x CBA) fertilized oocytes. 3) The resulting offspring was analysed for the presence of the transgene by Polymerase Chain Reaction using primers specific for the hG6PDH gene and that do not hybridize to the homologous mouse G6PDH gene. Three founders capable of transmitting the transgene to the progeny were identified and subsequentially three congenic lines were established (hG6PDH-tg line 1, 2 and 3) by backcrossing the corresponding founders with inbread C57BL/6J mice in order to obtain almost pure (>99%) C57BL/6J hG6PDH-tg mice. Mice were generated at the Spanish National Cancer Research Center (CNIO, Madrid, Spain) at the Transgenic Mice core facility. After analysing the G6PDH overexpression level achieved in the three G6PDHtg lines established (hG6PDH-tg lines 1, 2 and 3) at the mRNA and protein level, we observed that G6PDH is moderately overexpressed (2-5 fold) in all tissues tested (including liver, lung, heart, muscle, white adipose tissue, kidney, spleen, brain and red blood cells) in the G6PDHtg line 1 and 2 only, whereas no G6PDH overexpression was observed in the G6PDHtg line 3. In this work, G6PDH-tg line 1 has been used. Some mice were sent at the medicine university of Valencia (Spain) to extend the colony and realize experiments while others were sent in Rennes only for experiments.

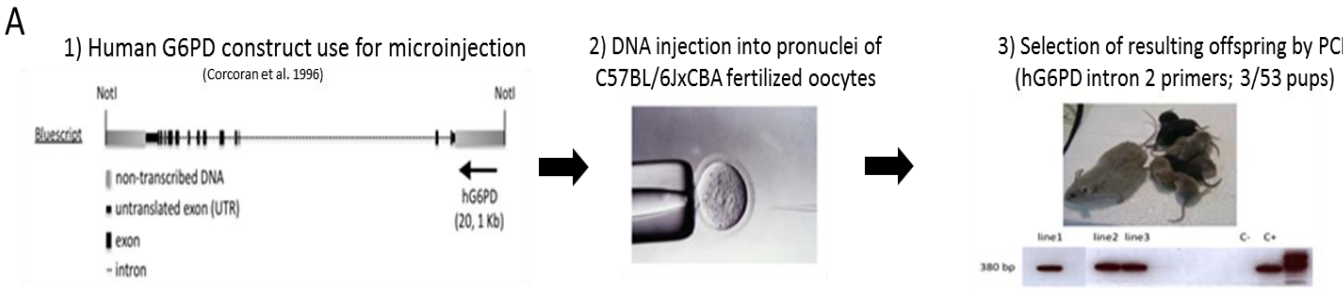


Figure 1. Generation of a human G6PDH-transgenic (hG6PDH-tg) mouse model

Animal care

This study was carried in three different research centers: Spanish National Cancer Research Center (CNIO, Madrid, Spain); Physiology department of the Medicine University of Valencia (Valencia, Spain) and the Movement, Sport and Health laboratory (Rennes, France). To avoid variation due to animal care, housing conditions were normalized in the same way in the three research centers.

The animals were always fed a normal rat chow (A.04; Panlab, Barcelona, Spain) and had free access to tap water. Mice were housed in a temperature-controlled room (24±2°C) with a light-dark cycle (12:12 h) until reach 12-14 months that was matched for the different experiments of the study. Only mal mice were used.

The study was conducted following recommendations from the institutional animal care and use committee, according to the Guidelines for Ethical Care of Experimental Animals of the European Union. Experiments were always approved by the local committee of ethics where was carried the experiment: the CNIO-ISCIII Ethics Committe for Research and Animal Welfare (CEIyBA), the Committee on Ethics in Research of the Medicine University of Valencia and the Committee on Ethics in Research of the University Rennes 1.

Body composition and muscle weights

At the aged of 12-14 months, lean mass (LM) and fat mass (FM) of individual mice (n = 10 per group) were quantified using dual energy X-ray absorptiometry (DXA) at the CNIO and normalized relative to body weight measured just before. LM and FM of individual mice (n = 10 per group) were also evaluated by bio-impedancemetry in Rennes to validate this technic in mice but data are still under analysis. The weights of individual muscles were measured after each done sacrifice in the different research centers following a meticulous

dissection (trimming away the excess connective and adipose tissue) done by the same examiner in the same conditions. Muscles were always directly frozen in liquid nitrogen, and stored at -80°C until analysis. Data presented are these about one representative sacrifice carried with 10 animals of each group (G6PDHtg and WT). However, nevertheless the place where was done the sacrifice, statistical analyses gave always the same result in each muscle weighted (see figure 4).

Spontaneous activity

Spontaneous activity was approached by measuring activity of mice on running wheels. Mice were housed in individual cage within a running wheel during a 4 weeks period (n=8 per group). Activity was monitored by a magnetic switch affixed to each wheel, which recorded the number of completed revolutions. Physical activity was recorded continuously and summed by days for analysis. The ten first days were considered as an acclimation period and data started to be included in the analysis only since the 11th day.

Dietary study

In the way to study food consumption, diuresis and feces, mice (n=5) were placed in special cage (as on the following photo) allowing measuring each of the aforementioned parameters, during a 4 weeks period. The ten first days were considered as an acclimation period and data started to be included in the analysis only since the 11th day. Data were daily recorded.

Aerobic qualities assessments

The exercise testing protocol was performed on a single-lane motorized treadmill (Panlab, Spain) with an adjustable belt speed (0–99.9 m.min-1). The rear of the treadmill was equipped with a low-voltage, electric stimulating bar, to encourage each mouse to run. The bar was set to deliver 0.2 mA at a frequency of 0.25 Hz, which caused an uncomfortable shock but did not injure the animal.

Measurements and data recording

Oxygen consumption (VO2) was measured by means of a rapid-flow, open-circuit, indirect calorimeter. The single-lane test treadmill was placed in a metabolic chamber. Ambient air was fed through the chamber at a rate of 0.66 $l.min^{-1}$; the flow was chosen such that the O_2 difference across the chamber was within the sensor's range (-0.5 to -0.8% O_2). A fan mixed the incoming air with the air around the treadmill and blew it towards the animal (LE 4002FL, Panlab, Spain). The air flowed from the front of the treadmill to the rear and then returned under the belt towards the front. This created a rapid, circular "loop" of mixed gases (incoming "fresh" air and accumulated exhaled gases) from which a sample was drawn for analysis (LE 405 O_2/CO_2 Analyzer, Panlab, Spain). Gas samples were taken every 5s and dried prior to measurement of the oxygen and carbon dioxide fractions.

The gas analyzers were calibrated with standardized gas mixtures (Linde AG, Paris, France) before every test session, as recommended by the manufacturer. The treadmill test provided an estimate of VO2max, defined as the highest oxygen consumption attained over a 15-second period during the testing protocol. To allow rapid comparison over a wide range of body weights (and especially with human data), dimensional analysis and empirical studies show that VO_2 should be expressed in relation to body mass raised to the power of 0,75 (Taylor et al. 1981; Mille-Hamard et al. 2012).

Familiarization

As previously described (Mille-Hamard et al. 2012), the mice were familiarized with the treadmill over a one week period *via* the completion of four 10min running sessions from 0 to 9 $m.min^{-1}$ (0, 3, 6 and 9 $m.min^{-1}$). All mice succeeded in running for the required time at

an intensity of 9m.min^{-1}. The velocity was not increased above this value, in order to avoid a training effect. The mice subsequently performed an incremental exercise test.

Incremental test load: VO2max determination

Starting from a speed of 10 m.min^{-1}, the exercise intensity was increased by 3m.min^{-1} every 2 min, with an incline of 15%. This protocol was used because it has been described as the test providing the higher VO_2max in mice (Høydal et al. 2007). Exercise continued until exhaustion, which was defined as an inability to maintain the running speed despite contact with the electric grid for more than 5 sec (Mille-Hamard et al. 2012). Exhaustion was then confirmed during the data analyses by the VO_2max stagnation while speed was still increased. All measurements were made by the same investigator. The last stage completed by the mouse was defined as the peak velocity (vPeak).

Endurance capacity determination

Endurance capacity was assessed using a modified protocol from Brooks & White (1978), at least 48 hours after maximal oxygen uptake determination to avoid interferences between the two tests (Mille-Hamard et al. 2012). Starting from a speed corresponding to 50% of vPeak, the exercise intensity was increased by 5% of vPeak every 2 min until reach 75% of vPeak, with an incline of 15%. At this moment, mice ran at this speed until exhaustion which was defined as an inability to maintain the running speed despite contact with the electric grid for more than 5 sec (Mille-Hamard et al. 2012). During this test, gas exchanges were not measured.

Muscle performance assessment

On the one hand, grip strength test was assessed as previously described to determine the maximum grip strength of the front legs (Pareja-Galeano et al. 2012). Briefly, for acclimating (3 preceding days, no more to avoid learning) and testing, mice (n = 10 per group) grasped a bar linked to an electronic dynamometer (GS3®, Bioseb) with their front limbs and were then drawn away from the bar by the same examiner. Five trials were performed with 8 minutes of rest between trials to allow a complete recovery. The average peak force of the five trials was normalized to body weight and used for subsequent analyses.

On the other hand, mesh grip suspension test was used to determine muscle fatigue as described previously (Dunn & Pinkert 2012). Briefly, for acclimating (3 preceding days, no more to avoid learning) and testing, mice (n = 10 per group) are placed on a grid, located about 50 cm above a foam padded cage to prevent injury. Then, when mice were well gripped, the metal grid was gently returned by the same examiner. From this point, the stopwatch is started until the animal fall. The test is repeated three times at 20 minute intervals to allow a complete recovery. The average time of the three trials was normalized to body weight and used for subsequent analyses. This test has been done in two different research centers (Valencia and Rennes) with the same examiner with different animals of the same age and statistical analysis gave always the same results.

Determination of glucose-6-phosphate dehydrogenase (G6PDH) activities in gastrocnemius muscle, liver and erythrocytes

Glucose-6-phosphate activity was determined using the techniques of Waller and co-workers (1986). Briefly, 1000 µl of glucose-6-phosphate (final []=10 mM) in potassium phosphate buffer was added to a cuvette. Then 400 µl of muscle or liver homogenate or erythrocyte lysate were introduced. The reaction was initiated by 400 µl of NADP (final []=0.90 mM) in buffer. The mixture was inverted and the absorbance read over 3 min at 340 nm using a spectrophotometer. Results were obtained in nmol x mg of protein^{-1} x min^{-1}. Values were then normalized to those observed in the samples obtained from the WT group, which were assigned a value of 100%. Protein concentrations were determined by Bradford's method (Bradford 1976) by using bovine serum albumin as standard.

Gastrocnemius and Liver DNA content

DNA was extracted from an entire gastrocnemius muscle and a piece of liver *via* the High Pure PCR Template Preparation Kit (Roche, GmbH, Germany) according to the manufacturer's protocol. DNA concentrations were measured *via* a plate spectrophotometer for nucleic acids (ND-2000, NanoDrop, Wilmington, DE). All analyses were done in triplicate. Total gastrocnemius or liver DNA was calculated from DNA concentration normalized to whole muscle wet weight or liver piece wet weight.

Plasmatic uric acid level

During sacrifice, blood was collected by venous puncture (inferior cava venous and processed to measure plasma uric acid. Uric acid concentration assessment was carried into a Randox Daytona automate using a commercial kit (URIC ACID Liquid Mono Reagent, COLORIMETRIC RX Daytona^{TM}, Randox, France) according to the manufacturer's protocol.

Immunoblot analysis

Aliquots of muscle lysate (50-120 µg of proteins) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The whole gastrocnemius was used to ensure homogeneity. Proteins were then transferred to nitrocellulose membranes, which were incubated overnight at 4 °C with appropriate primary antibodies: anti-phosphorylated p70S6K (1:1000, Cell Signaling); anti-G6PDH (1:1000, Abcam, UK); anti-AKT (1:1000, Cell Signaling); anti-phosphorylated AKT (1:1000, Cell Signaling); anti-PPARα (1:200, Santa cruz Biotechnology, CA), anti-CPT I (1:200, Santa cruz Biotechnology, CA), anti-Cytochrome C (1:200, Santa cruz Biotechnology, CA); anti PGC-1α (1:1000, Cell Signaling) and anti-MHC (1/1000, Santa Cruz Biotechnology, CA). Thereafter, membranes were incubated with a secondary antibody for 1 h at room temperature. Specific proteins were visualized by using the enhanced chemiluminescence procedure as specified by the manufacturer (Amersham Biosciences, Piscataway, NJ). Autoradiographic signals were assessed by using a scanning densitometer (BioRad, Hercules, CA). Data were represented as arbitrary units of immunostaining. To check for differences in loading and transfer efficiency across membranes, an antibody directed against α-actin (1:1000, Sigma Aldrich Missouri) was used to hybridize with all the membranes previously incubated with the respective antibodies. For the Western Blotting quantifications we first normalized all the proteins measured to α-actin. Samples from each group were run on the same gel.

Statistical Analysis

Statistical analyses were performed using the SigmaStat 3.1 Program (Jandel Corp., San Rafael, CA). Results are expressed as mean ± SD. Normality of distribution was checked with the Kolmogorov test and homogeneity of variance was tested by Levene's statistics. We used a student-test to compare group differences. Differences were considered significant if $p<0.05$.

RESULTS

G6PDH overexpression increases G6PDH activity and protein content

To confirm that G6PDH overexpression was effective, we measured G6PDH activity in various tissues and protein content especially in skeletal muscle because it was the tissue of interest in this work. Results are shown in Figure 2. G6PDH overexpression multiplied G6PDH activity by 1,5 in erythrocytes (see panel A, $p<0,01$), by 2 in gastrocnemius muscle (see panel B, $p<0,01$) and by 4,5 in liver (see panel C, $p<0,01$) in G6PDHtg mice compared to WT mice. G6PDH gastrocnemius protein content was twofold higher in G6PDHtg mice compared to WT mice (see panel D, $p<0,01$).

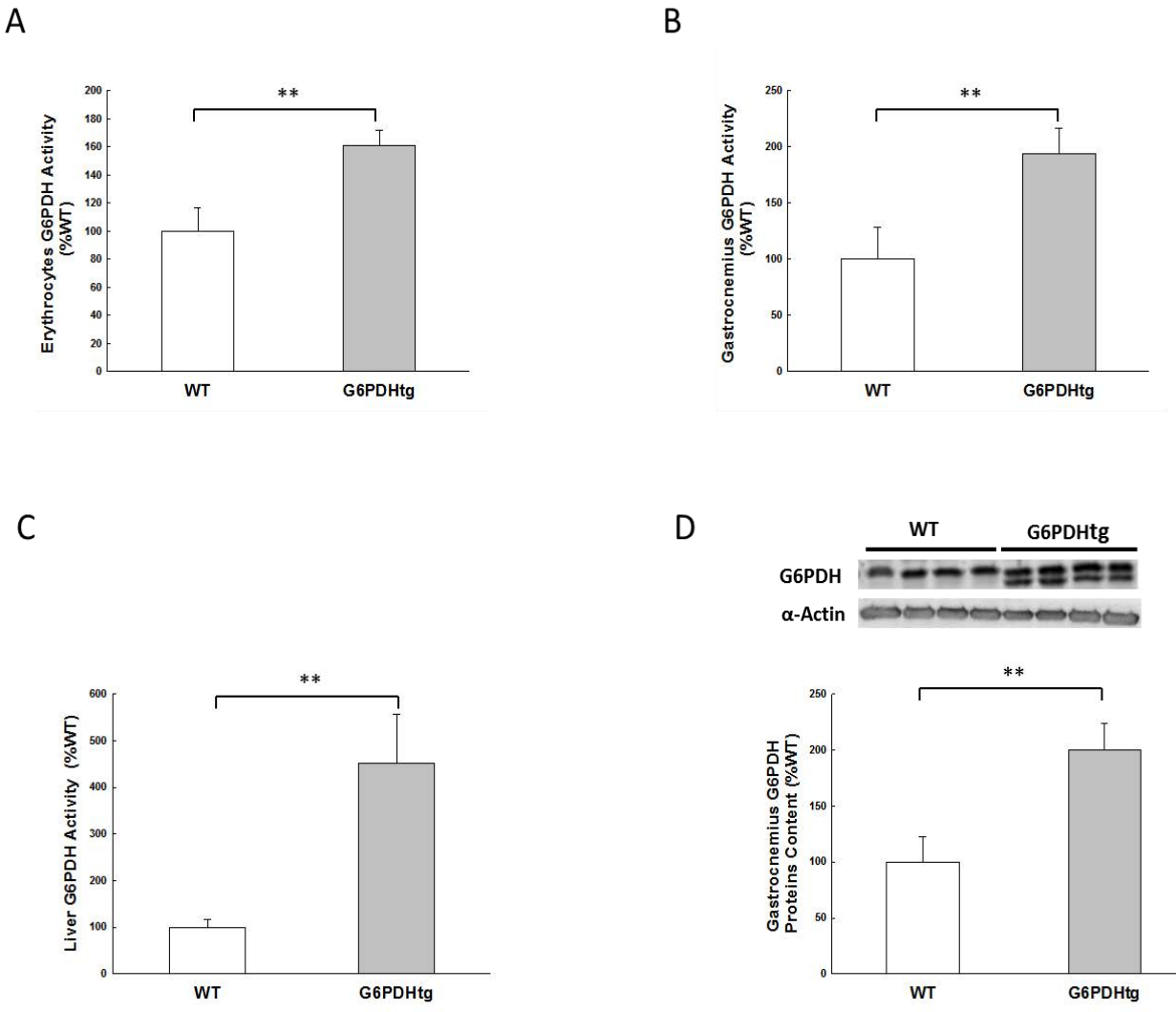


Figure 2. G6PDH overexpression increases G6PDH activity and protein content.

Animals were divided into two experimental groups: Wild type (WT) (n=10) and G6PDH transgenic mice (G6PDHtg) (n=10). Panels A, B and C show G6PDH enzymatic activity respectively in erythrocytes, gastrocnemius muscle and liver. Western blotting analysis to detect G6PDH (Panel D) in mice gastrocnemius muscle was performed. Representative blots are shown. The content of α-actin, a housekeeping protein marker in skeletal muscle, was determined in all the experimental groups. Values were normalized to those observed in the samples obtained from the WT group, which was assigned a value of 100%. Values are shown as mean (±SD). *p<0,05.

G6PDH overexpression leads to a slightly muscle mass increase

To confirm data suggesting that G6PDH would be implicated in muscle mass development (Max 1984; Kovacheva et al. 2010), we achieved a body composition study. First, animals were weighted and we observed that G6PDHtg mice were thinner than WT mice (see figure 3, panel A, p<0,01). Lean mass (LM) and fat mass (FM) were measured by DXA and corrected by the animal body weight. Bone mineral density (BMD) was also assessed by DXA. Our results showed that G6PDH overexpression led to an increased LM (G6PDHtg + 13,8% *vs* WT, p<0,01.) and a decreased FM (G6PDHtg - 13,8% *vs* WT, p<0,01) as shown respectively on the figure 3, panels B and C. Increased lean mass was attributed due to an increased muscle mass since the BMD was similar in the two groups (data not shown).

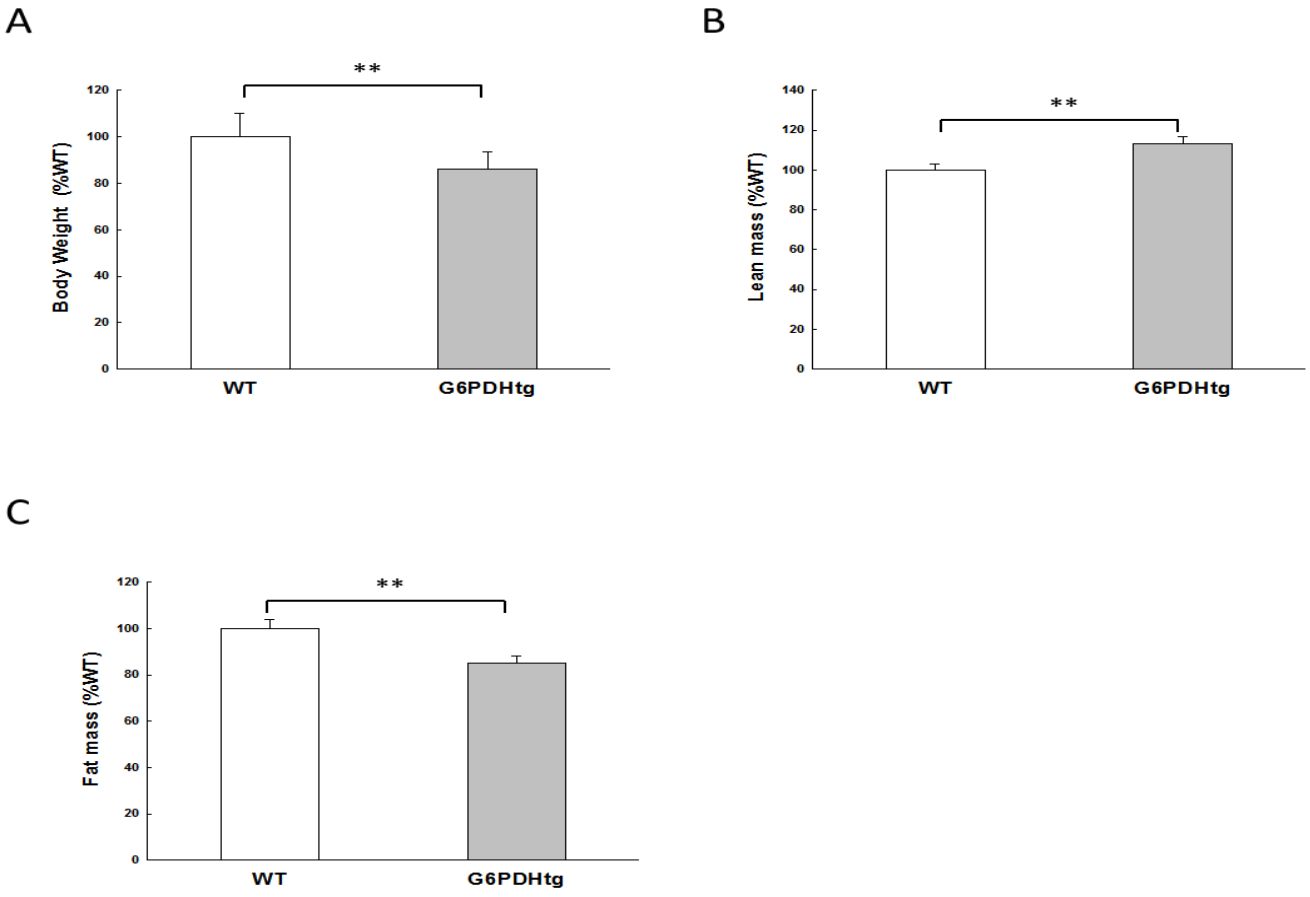


Figure 3. G6PDH overexpression improves body composition.

Animals were divided into two experimental groups: Wild type (WT) (n=5) and G6PDH transgenic mice (G6PDHtg) (n=5). Panel A shows animals body weight. Lean mass (Panel B) and fat mass (Panel C) of individual mice were quantified DXA and normalized relative to body weight. Values were normalized to those observed in the samples obtained from the WT group, which was assigned a value of 100% and are shown as mean (±SD). **p<0,01.

To complete and confirm these results, several skeletal muscles were weighted *post mortem* and corrected by the animal body weight. Figure 4, showed that tibialis (panel A), soleus (panel B) and gastrocnemius (panel C) muscle weight to body weight ratios were 13%, 12% and 14% greater (p<0,05 in all cases) in G6PDHtg mice compared with WT mice respectively. Others organs such as liver, kidney and heart were also weighted and the lack of difference between WT and G6PDHtg confirmed that only skeletal muscle were hypertrophied. In order to confirm muscle hypertrophy and assess muscle quality, gastrocnemius total MHC was measured by western blotting (see panel D). G6PDHtg mice presented higher MHC protein content in gastrocnemius muscle than WT mice (+12%, p<0,01).

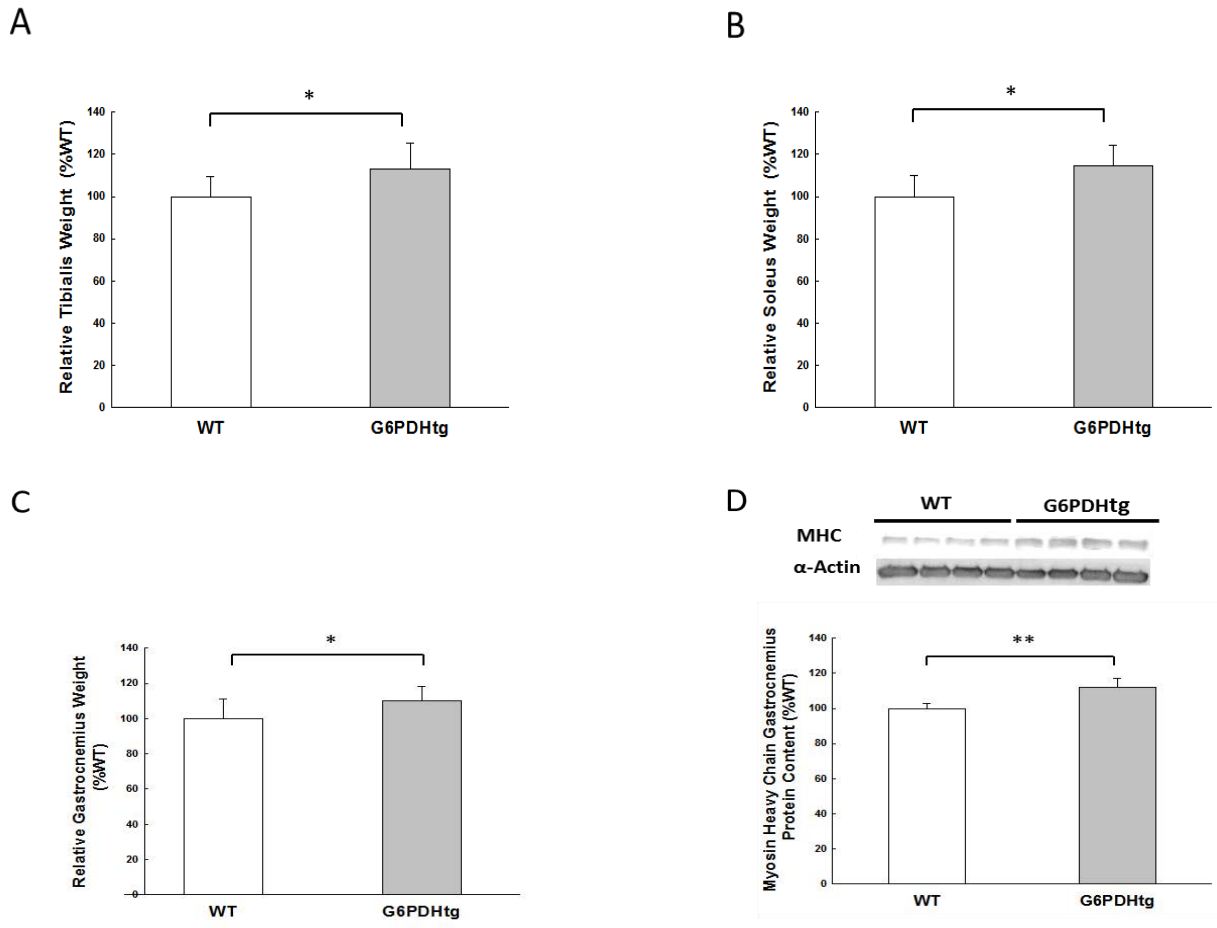


Figure 4. G6PDH overexpression leads to a slightly hypertrophic phenotype.

Animals were divided into two experimental groups: Wild type (WT) (n=10) and G6PDH transgenic mice (G6PDHtg) (n=10). Panel A, B and C show respectively tibialis, soleus and gastrocnemius muscle weight to body weight ratios. Western blotting analysis to detect total MHC (Panel D) in mice gastrocnemius muscle was performed. Representative blots are shown. The content of α-actin, a housekeeping protein marker in skeletal muscle, was determined in all the experimental groups. Values were normalized to those observed in the samples obtained from the WT group, which was assigned a value of 100%. Values are shown as mean (±SD). *p<0,05 ; **p<0,01

Although muscle mass and strength are linked, several studies have shown that the relation is not linear (Goodpaster et al. 2006; Janssen 2004) and consequently a higher muscle mass does not guarantee greater strength. Thus, muscle strength was evaluated in two ways (figure 5). G6PDH overexpression was associated with a significantly 10 percent higher voluntary grip force (see panel A, p<0,05) and significantly 88 percent higher time latency before falling in the mesh grip strength test (see panel B, p<0,05).

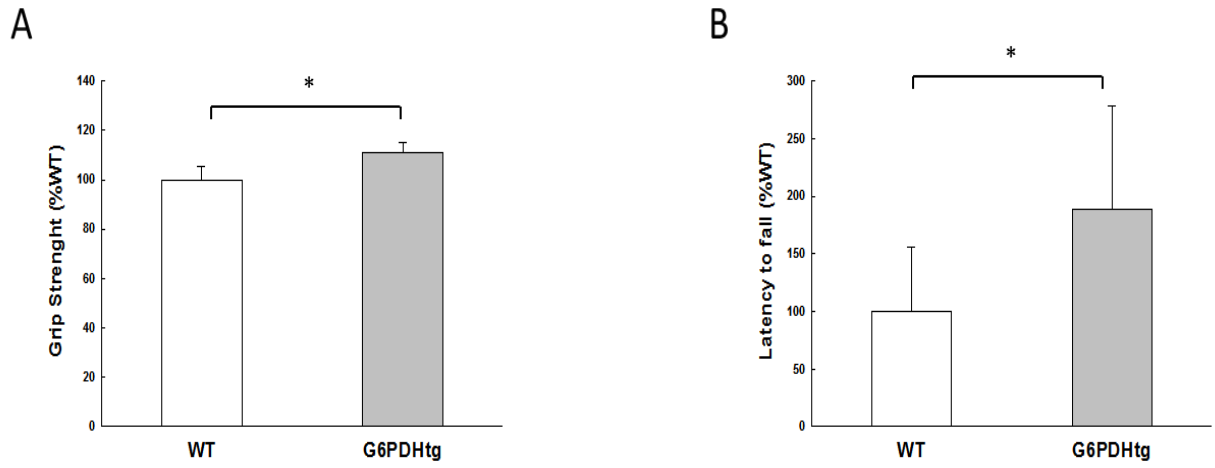


Figure 5. G6PDH overexpression improves muscle performance.

Animals were divided into two experimental groups: Wild type (WT) (n=10) and G6PDH transgenic mice (G6PDHtg) (n=10). Panel A shows results of the grip strength test. Panel B presents latency to fall during the mesh grip strength test. In both tests, values were normalized to body weight. Values were normalized to those observed in the samples obtained from the WT group, which was assigned a value of 100%. Values are shown as mean (±SD). *p<0,05.

Muscle hypertrophic profile associated with the overexpression of G6PDH is not due to an alteration of dietary behavior or spontaneous activity.

To ensure that changes in body composition were due to biomolecular changes and not to behavioral alterations, the spontaneous activity of mice as well as feeding behavior were studied during two weeks. Whatever the parameter studied, overexpression of G6PDH did not induce changes. Thus, daily food and water intake, daily urine and feces were similar between G6PDHtg and WT mice (see respectively panels A, B, C and D).

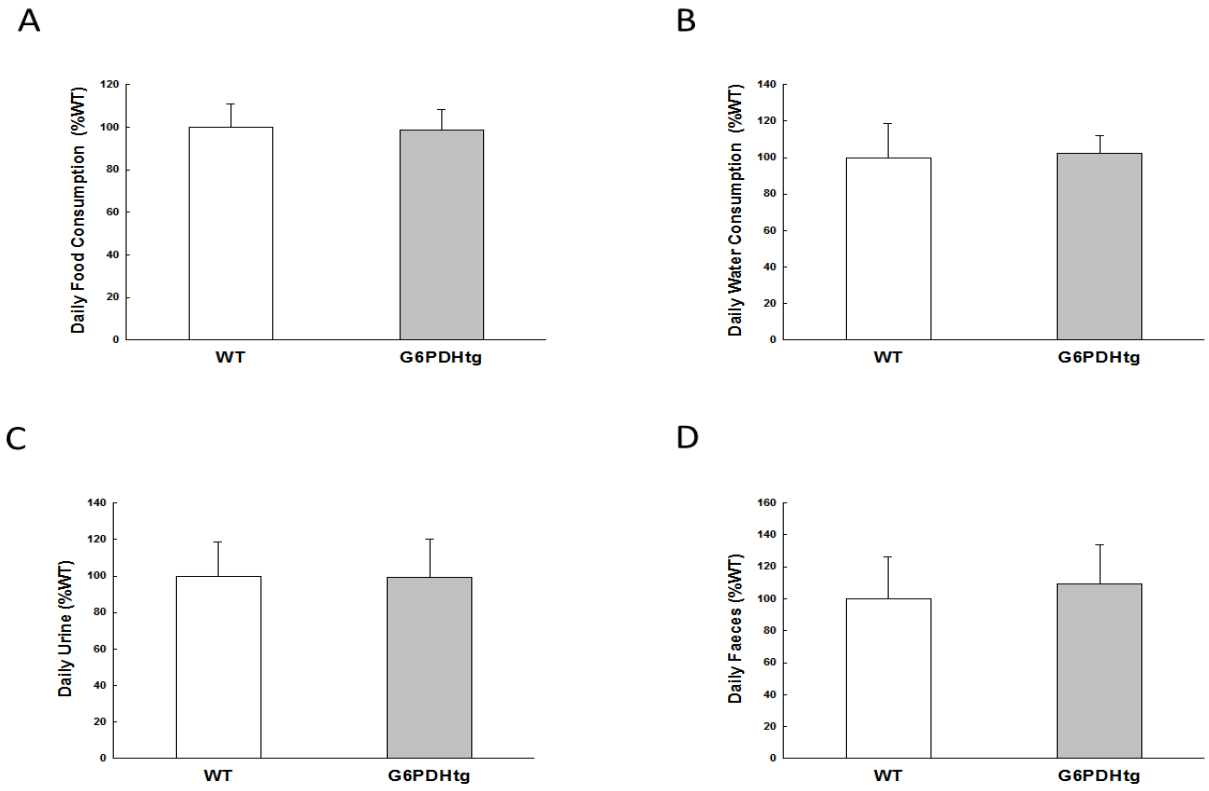


Figure 6. G6PDH overexpression does not alter dietary behavior, daily urine and feces. Animals were divided into two experimental groups: Wild type (WT) (n=5) and G6PDH transgenic mice (G6PDHtg) (n=5). All the values were normalized to body weight. Values were normalized to those observed in the samples obtained from the WT group, which was assigned a value of 100%. Values are shown as mean (±SD).

Moreover, daily running distance and time were comparable into the two groups (see respectively panel A and B of figure 7).

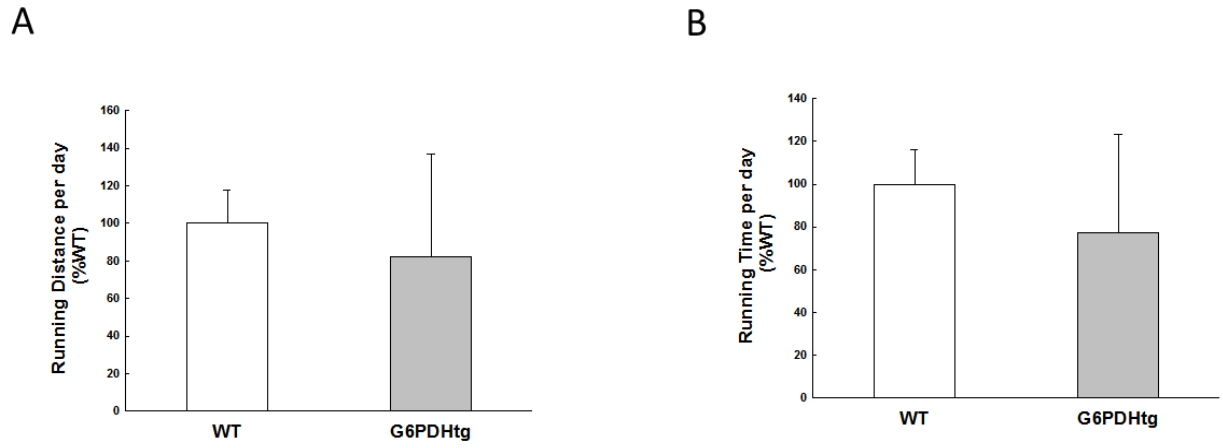


Figure 7. G6PDH overexpression does not alter spontaneous activity.
Animals were divided into two experimental groups: Wild type (WT) (n=8) and G6PDH transgenic mice (G6PDHtg) (n=8). Panel A represents values of running distance per day. Panel B show the daily running time. Values were normalized to those observed in the samples obtained from the WT group, which was assigned a value of 100%. Values are shown as mean (±SD).

Overexpression of G6PDH appears to increase nucleic acids metabolism without change in protein synthesis signaling pathway

In numerous cell lines (but not in muscle cells), overexpression of G6PDH accelerates proliferation due to an increased DNA, RNA and protein synthesis (Tian et al. 1998; Kuo et al. 2000). By measuring in gastrocnemius DNA content and protein expression of several markers of protein synthesis and uric acid plasma several, we tried to confirm this data in our mice overexpressing G6PDH (see figure 8). Here, G6PDHtg mice showed a significantly higher DNA gastrocnemius DNA content compared to WT mice (Panel A ; $p<0,01$). Similar results were obtained in liver ($p<0,01$; data not shown). Uric acid (UA) is the terminal product of purine metabolism and could be measured as an indirect marker of cell turnover and consequently of DNA turnover (Banfi & Colombini 2012). We found that G6PDHtg mice have a two folds higher plasmatic UA concentration than WT mice (Panel B ; $p<0,01$). From a theoretical point of view, the amount of DNA is a critical determinant of protein synthesis capacity by providing necessary to sustain gene transcription. To have an idea of a possible improvement in protein translation, two essential components of the PI3K/Akt/mTOR

pathways controlling protein translation were measured by western blotting. However, our result failed to show any change between the two groups (Panels C and D).

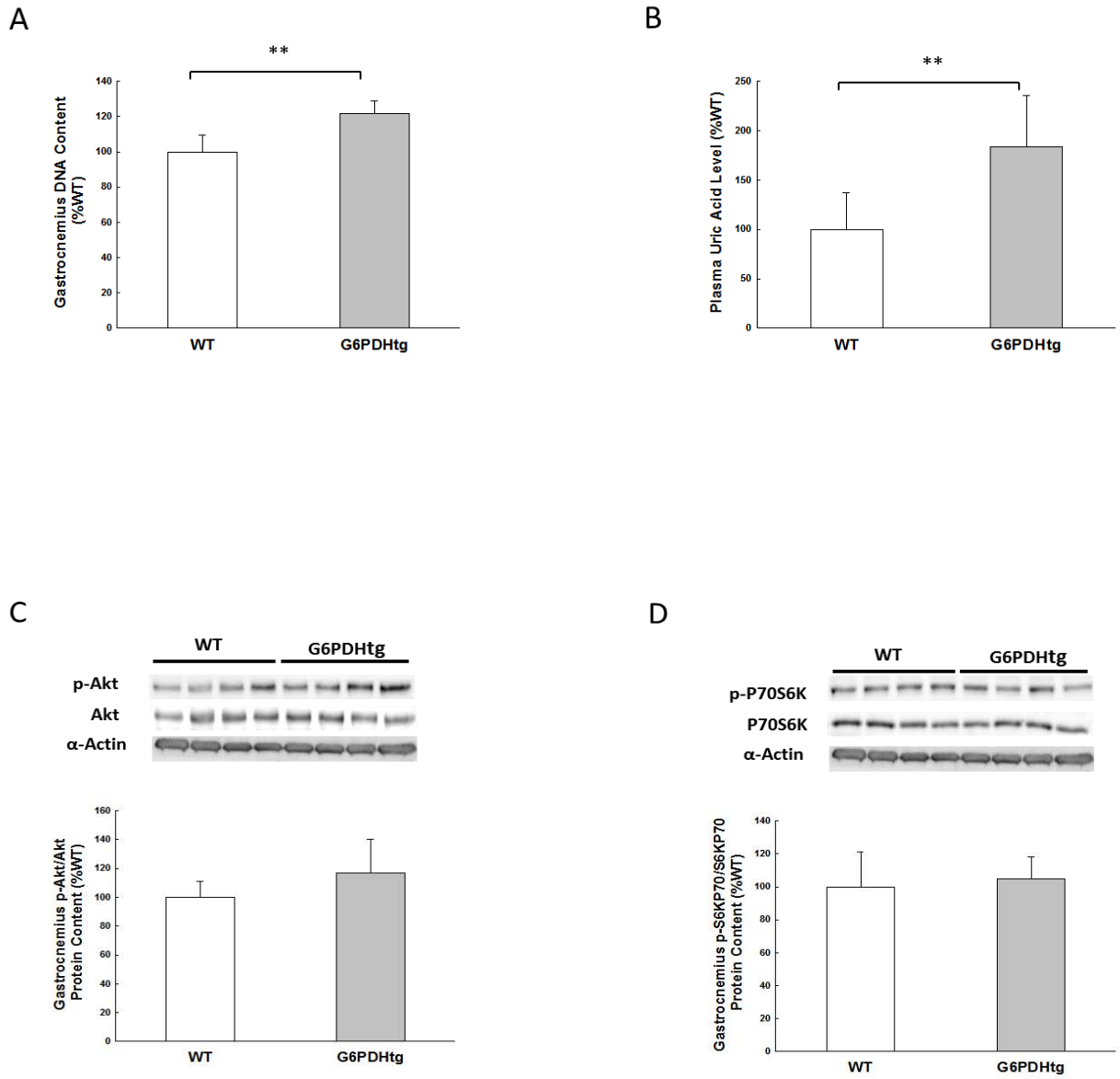


Figure 8. G6PDH overexpression increases gastrocnemius DNA content, uric acid plasma without any change in main components if the PI3K/Akt/mTOR pathway.

Animals were divided into two experimental groups: Wild type (WT) (n=8) and G6PDH transgenic mice (G6PDHtg) (n=8). Panel A represents values of gastrocnemius DNA. Panel B shows plasma uric acid concentration. Western blotting analysis to detect Akt and its phosphorylated form (Panel C), p70S6K and its phosphorylated form (Panel D) in mice gastrocnemius muscle was performed. Representative blots are shown. The content of α-actin, a housekeeping protein marker in skeletal muscle, was determined in all the experimental groups. Values were normalized to those observed in the samples obtained from the WT group, which was assigned a value of 100%. Values are shown as mean (±SD). **p<0,01.

G6PDH overexpression is associated with an increased maximal oxygen uptake

In order to complete the characterization of our model, aerobic qualities were evaluated. Results are presented in the figure 9. We found that VO2max of G6PDHtg mice was significantly higher compared to WT mice (+12% ; p<0,05). However, absolute VO2max values (ml/min) were not different (data not shown). On the other hand, endurance capacity measured as the exhaustion time during running at 75% of vPeak was unchanged between the two groups.

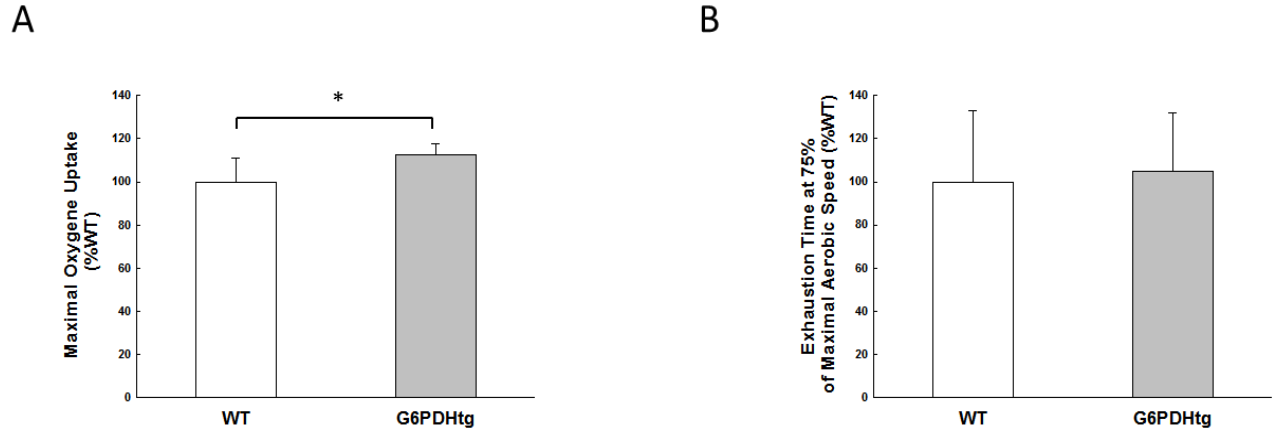


Figure 9. G6PDH overexpression improves aerobic qualities.
Animals were divided into two experimental groups: Wild type (WT) (n=10) and G6PDH transgenic mice (G6PDHtg) (n=10). Panel A shows maximal oxygen uptake. Data were first expressed in relation to body mass raised to the power of 0,75 and then were normalized to those observed in the samples obtained from the WT group, which was assigned a value of 100%. Panel B reports exhaustion time during a test at 75% of the vPeak. Values are shown as mean (±SD). *p<0,05.

Improved maximal oxygen uptake with the overexpression of G6PDH would not be due to an increased lipids catabolism

To determine whether differences in VO2max could be explained by others reasons than weight differences, we decided to explore important markers involved in lipids catabolism and miochonriogenesis. Peroxisome proliferator-activated receptors α (PPARα; which regulates the expression of proteins involved in fatty acid oxidation, Panel A), carnitine palmitoyltransferase I (CPT I ; catalyzes the first reaction in the transport of longchain fatty

acids from the cytoplasm to the mitochondrion, a rate-limiting step in β-oxidation, Panel B), peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 α; master regulator of mitochondriogenesis, Panel C) and cytochrome C (used as a marker for mitochondrial density, Panel D) were measured by western blotting in gastrocnemius. The results are presented in the figure 10. PPARα and CPT I, as well as, PGC-1 α and cytochrome C gastrocnemius protein content were similar between G6PDHtg and WT mice.

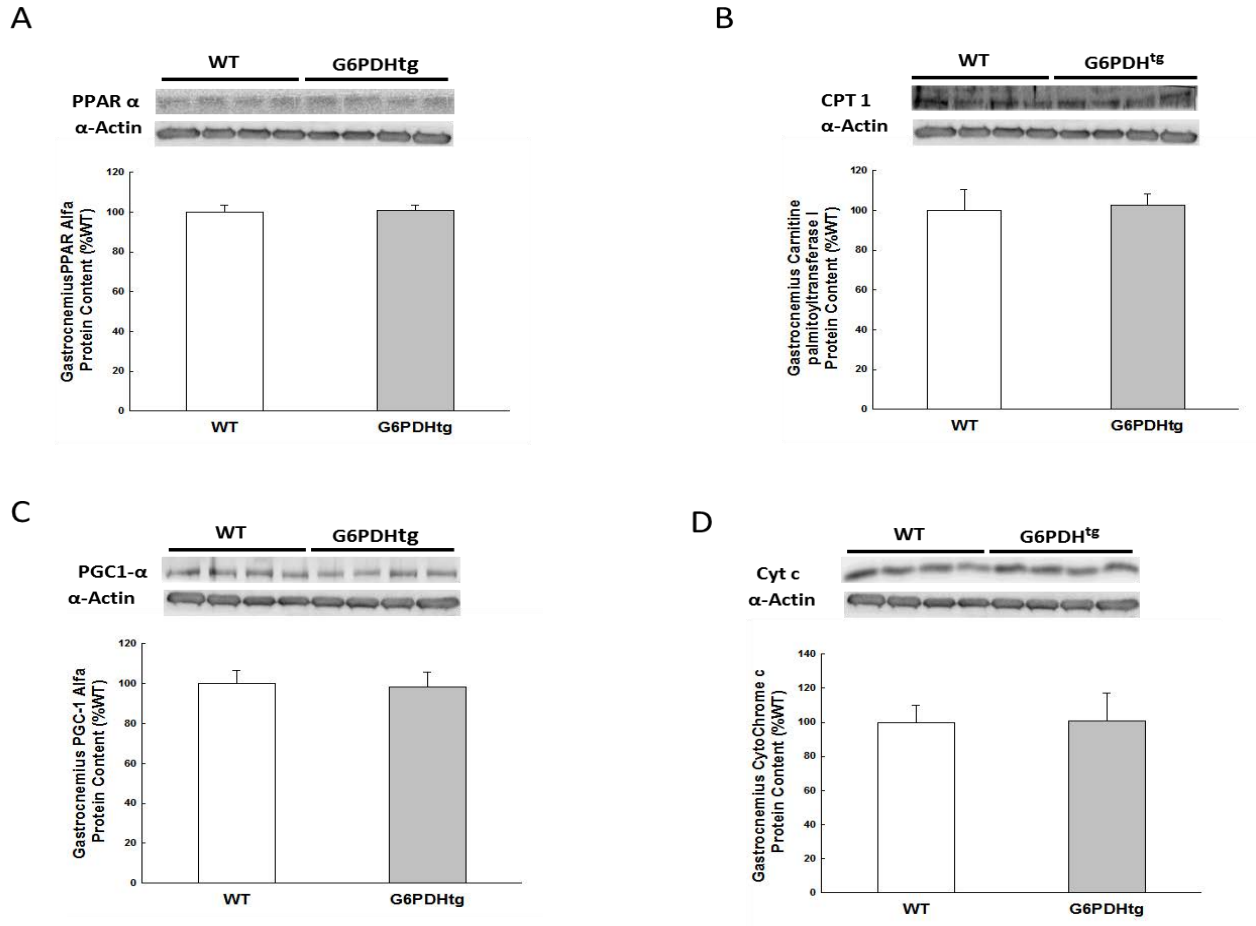


Figure 10. G6PDH overexpression, lipids catabolism and mitochondriogenesis markers. Animals were divided into two experimental groups: Wild type (WT) (n=8) and G6PDH transgenic mice (G6PDHtg) (n=8). Western blotting analysis to detect peroxisome proliferator-activated receptor α (PPARα ; Panel A), carnitine palmitoyltransferase I (CPT ; Panel B), Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α ; Panel C) and cytochrome C (Cyt c; Panel D) in mice gastrocnemius muscle was performed. Representative blots are shown. The content of α-actin, a housekeeping protein marker in skeletal muscle, was determined in all the experimental groups. Values were normalized to those observed in the samples obtained from the WT group, which was assigned a value of 100%. Values are shown as mean (±SD).

DISCUSSION

Our study is the first one which found in mice that G6PDH overexpression improved body composition by decreasing fat mass and increasing muscle mass surely due to an increase in the protein synthesis capacity through an increase in muscle DNA content. Improvement in body composition was associated with better muscle strength and aerobic qualities.

First, we confirmed that G6PDH overexpression was effective in the G6PDHtg mice by measuring its activity and protein expression in several tissues and notably in skeletal muscle where the activity and the protein content were two fold higher compared to WT mice. These data are in accordance by the previous data published by Corcoran et al. (1996) which were the only group working on these transgenic mice before us. As they published, we found that overexpression lead to an increased G6PDH activity which varies from one tissue to another. Indeed, while G6PDH activity was 2 fold higher in skeletal muscle, we found that it was multiplied by 1,5 in erythrocytes and by 4,5 in liver.

Improvement of body composition and notably increased muscle mass would be very important in sarcopenia which is characterized by a decreased muscle mass and strength (Cruz-Jentoft et al. 2010). Moreover, decreased fat mass would have also beneficial effect on muscle function. Indeed, during sarcopenia there is an age-related infiltration into skeletal muscle by fat associated with an increased adipogenesis, which is a powerful predictor of future disability and mortality (Visser et al. 2005). We reported that overexpression of G6PDH lead to a lower body weight associated with an increased lean mass percentage and a decreased fat mass percentage (see figure 2). This higher lean mass was due to an increased muscle mass for the following reasons. Lean body mass is composed of muscles, bones, and internal organs. In our case, since the bone mineral density (which can be used as an indirect reflect of the bone mass) was similar between the G6PDHtg and WT mice, increased bone mass to explain the increase in lean mass was excluded. In the same way, since internal organs, such as heart, liver and kidney weight normalized to body weight were not different in the two groups (data not shown), increased lean mass due to an increase in internal organs weight was also excluded. Finally, the unique difference in the compounds of lean mass was found for muscle weight normalized to body weight. Indeed, for the three muscle weighted (soleus, gastrocnemius and tibialis anterior), we found that the muscle weight/body weight ratios were higher in the G6PDHtg mice compared to the WT mice. For these different reasons, we concluded that increased lean mass was due to an increased muscle mass.

Increased MHC protein in skeletal muscle of the G6PDHtg mice compared to the WT mice confirmed the increase in muscle mass in these latter.

These results proved that G6PDH plays a central role in muscle mass regulation as it was supposed by several studies. Indeed, Max (1984) and Kovacheva et al. (2010) showed that muscle atrophy was associated with decreased G6PDH activity and protein content while muscle hypertrophy was associated with increased G6PDH activity and protein content. Muscle regeneration was also associated with increased G6PDH activity (Wagner et al. 1977; Wagner et al. 1978). It was proposed that an increased G6PDH activity would lead to augment ribose-5-phosphate (R5P) synthesis leading to an increase in DNA and RNA and protein. This hypothesis has been confirmed in vitro, where G6PDH overexpression led to an improved cell growth due to a higher DNA synthesis leading to an amplified protein synthesis (Tian et al. 1998; Kuo et al. 2000). However, it has been never confirmed in vivo. In our animal model, we found an increased gastrocnemius DNA content which confirmed the aforementioned studies. Otherwise, the higher uric acid plasma levels observed in G6PDHtg mice attested that G6PDH overexpression increased nucleic acid turnover. A higher DNA content would confer to the G6PDGtg mice a higher transcription capacity which would increase protein synthesis and lead to a higher muscle mass. However, we would directly measure protein synthesis to confirm this hypothesis. Protein synthesis is also controlled by translation which principally regulated by the PI3K/Akt/mTOR pathway. As increased G6PDH activity is associated with activation of the PI3K/Akt/mTOR pathways in vitro (Stanton 2012), we explored if the relation is present in vivo. In this way, we measured Akt and p70S6K which have been found to increase protein synthesis when activated in skeletal muscle (Kimball et al. 2002). Unfortunately, we did not shown any difference between G6PDHtg and WT mice. Finally, in regards to our actual results, the higher lean mass observed in response to G6PDH overexpression would be due to an increase protein synthesis thanks to a higher transcriptional capacity.

As previously described, our results showed that G6PDH overexpression led to an increase in lean mass. The later was associated with a decrease in fat mass. This decrease in fat mass was very surprising since an increase in G6PDH activity have been usually associated with a lipogenesis due to increased G6PDH activity in adipose tissue (Park et al. 2005b; Bonnet et al. 2007; Zomeño et al. 2010). G6PDH deficient mice have also been found to be protected against increased adiposity induced by high-fat/high-sugar diet (Hecker et al. 2012). However, the relation between increase in G6PDH activity and increase in lipogenesis is not always found (Bonnet et al. 2007). Indeed, these authors found in two different strains

of cattle that the lower rib fat thickness was observed in the strain with the higher mRNA levels and activities of G6PDH. Thus, the decreased fat mass observed in our study could be explained by a decreased lipogenesis and/or a higher lipids utilization. Indeed, Park et al. (2005) observed that G6PD-overexpressed adipocytes (3T3-L1 cells) significantly increased hormone-sensitive lipase expression elevating the levels of cellular free fatty acids, triglyceride, and free fatty acids release. We can therefore suppose that these free fatty acids will be uptaked by peripheric tissues such as skeletal muscle (Osterlund 2001; Lafontan & Langin 2009). In our case, we explored skeletal muscle markers involved in lipids catabolism (PPAR α and CPT I) without unfortunately found any change. These measures could be completed by measuring other markers such as the fatty acid transporter FAT/CD36 which is a key protein involved in regulating the uptake of free fatty acids across the plasma membrane in heart and skeletal muscle (Bonen et al. 2004). A decreased lipogenesis could be also envisaged in our model. Indeed, it has been shown that G6PDH is the main NADPH-producing enzyme but others have been identified such as malic enzyme (ME), and isocitrate dehydrogenase (ICDH). Merritt et al. (2009) have recently shown in flies that variations in one NADPH-producing enzyme can be met by reciprocal variations in the other enzymes. Indeed, when G6PDH was increased, it was associated with a lower activity ME and ICDH. This relation has also been shown in rats in liver, adipose tissue and skeletal muscle (Barakat et al. 1989; Lawler & Demaree 2001). Since ME plays an important role in lipogenesis, the lower ME activity by G6PDH decreased lipogenesis in adipocyte and liver. This relation will be explored in a next study.

Body composition can be influenced by physical activity and/or nutrition. The observed lack of difference between G6PDHtg and WT mice in nutritional behavior and spontaneous activity showed that neither could explain the improvement of body composition associated to G6PDH overexpression. Increased energy expenditure could not be also excluded to explain the improvement of body composition and notably the decreased in adiposity. Indeed, it has been shown a similar adiposity profile in another transgenic model presenting an increased basal energy expenditure (Ortega-Molina et al. 2012). However, these mice were more active compared to their WT counterpart. Moreover, it has been described a G6PDH activity in the small intestine (Hecker & Leopold 2013), a modification of nutrient absorption in response to G6PDH overexpression could not be excluded. However, no data have been published about G6PDH activity and nutrient absorption.

The improved body composition observed in the G6PDHtg mice was associated with better physical performances than those observed in WT mice. Indeed, we found higher muscle strength and higher maximal oxygen consumption. Body weight difference would principally explain these differences. Indeed, G6PDHtg mice were lower than the WT mice and only values normalized by the weight were higher in the G6PDHtg mice. It would be interesting to normalized strength and VO2max to lean mass values. In our case, it was not possible because mice using for the DXA analysis were not the same that those for strength tests. However, mice who performed physical tests were tested by BIA to assess their body composition but the values are still under validation.

In summary, we found in mice that G6PDH overexpression improved body composition by decreasing fat mass and increasing muscle mass surely due to an increase in the protein synthesis capacity through an increase in muscle DNA content. These data confirmed for the first time the suggested role of G6PDH in muscle mass regulation. These beneficial effects on body composition were associated with better muscle strength and aerobic qualities. Based on the results of this study, improving G6PDH activity would represent a good strategy to improve body composition and physical performance.

In a sarcopenic context, it would act as a double-edge sword, hypertrophic and lipolytic. In a more large vision, the beneficial effects observed in response to G6PDH overexpression, would lead to increase the health span of these mice and finally increase longevity as it has been shown in flies overexpressing G6PDH. These hypotheses are currently studied in the different teams who participated in this work.

Study 3: Redox status in resting conditions and in response to pro-oxidizing stimuli: impact of glucose-6-phospahe dehydrogenase overexpression

MATERIAL AND METHODS

Animals

Generation of a human G6PDH-transgenic (G6PDHtg) mouse model

In order to determine if a higher expression of G6PDH is effective in improving muscle mass, strength, physical performance and therefore improving health span in a vertebrate model, we decided to generate transgenic mice with a moderate overexpression of G6PDH under the control of its natural promoter (Figure 1). 1) For the generation of the G6PDH transgenic mice, a plasmid containing the human G6PDH (hG6PDH) entire genomic sequence (20,105 kilobases), including the entire upstream and downstream regulatory sequences, was used (Corcoran et al. 1996). 2) For transgenesis, the hG6PDH sequence was isolated from the pBluescript vector by NotI digestion and a 0,5 to 1 ng/ul DNA solution was injected into the pronuclei of F1 hybrids (C57BL/6J x CBA) fertilized oocytes. 3) The resulting offspring was analysed for the presence of the transgene by Polymerase Chain Reaction using primers specific for the hG6PDH gene and that do not hybridize to the homologous mouse G6PDH gene. Three founders capable of transmitting the transgene to the progeny were identified and subsequentially three congenic lines were established (hG6PDH-tg line 1, 2 and 3) by backcrossing the corresponding founders with inbread C57BL/6J mice in order to obtain almost pure (>99%) C57BL/6J hG6PDH-tg mice. Mice were generated at the Spanish National Cancer Research Center (CNIO, Madrid, Spain) at the Transgenic Mice core facility. After analysing the G6PDH overexpression level achieved in the three G6PDHtg lines established (hG6PDH-tg lines 1, 2 and 3) at the mRNA and protein level, we observed that G6PDH is moderately overexpressed (2-5 fold) in all tissues tested (including liver, lung, heart, muscle, white adipose tissue, kidney, spleen, brain and red blood cells) in the G6PDHtg line 1 and 2 only, whereas no G6PDH overexpression was observed in the G6PDHtg line 3. In this work, G6PDH-tg line 1 has been used. Some mice were sent at the medicine university of Valencia (Spain) to extend the colony and realize experiments while others were sent in Rennes only for experiments.

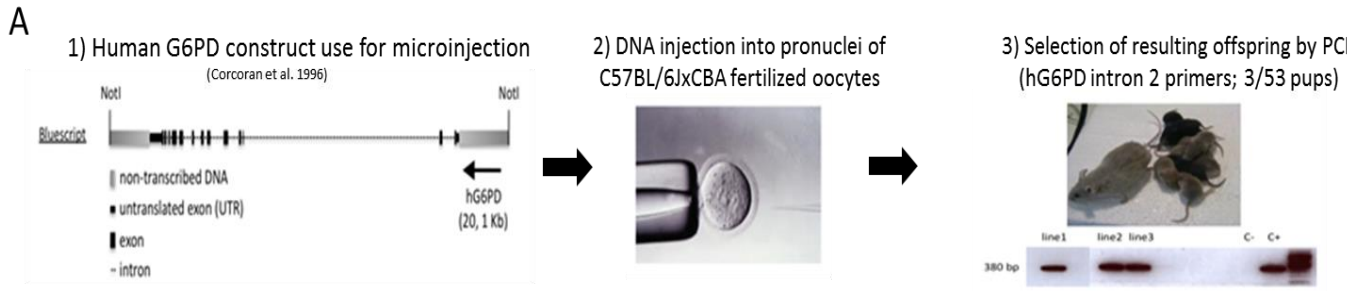


Figure 1. Generation of a human G6PDH-transgenic (hG6PDH-tg) mouse model

Animal care

The animals were fed a normal rat chow (A.04; Panlab, Barcelona, Spain) and had free access to tap water. Mice were housed in a temperature-controlled room (24±2°C) with a light-dark cycle (12:12 h) until reach 12-14 months that was matched for the different experiments of the study. Only male mice were used. The study was conducted following recommendations from the institutional animal care and use committee, according to the Guidelines for Ethical Care of Experimental Animals of the European Union. Experiments were always approved the Committee on Ethics in Research of the Medicine University of Valencia and the Committee on Ethics in Research of the University Rennes 1.

Exercise protocols

The exercise testing protocol was performed on a single-lane motorized treadmill (Panlab, Spain) with an adjustable belt speed (0–99.9 m.min-1). The rear of the treadmill was equipped with a low-voltage, electric stimulating bar, to encourage each mouse to run. The bar was set to deliver 0.2 mA at a frequency of 0.25 Hz, which caused an uncomfortable shock but did not injure the animal.

Measurements and data recording

Oxygen consumption (VO2) was measured by means of a rapid-flow, open-circuit, indirect calorimeter. The single-lane test treadmill was placed in a metabolic chamber. Ambient air was fed through the chamber at a rate of 0.66 $l.min^{-1}$; the flow was chosen such that the O_2 difference across the chamber was within the sensor's range (-0.5 to -0.8% O_2). A fan mixed the incoming air with the air around the treadmill and blew it towards the animal

(LE 4002FL, Panlab, Spain). The air flowed from the front of the treadmill to the rear and then returned under the belt towards the front. This created a rapid, circular "loop" of mixed gases (incoming "fresh" air and accumulated exhaled gases) from which a sample was drawn for analysis (LE 405 O_2/CO_2 Analyzer, Panlab, Spain). Gas samples were taken every 5s and dried prior to measurement of the oxygen and carbon dioxide fractions.

The gas analyzers were calibrated with standardized gas mixtures (Linde AG, Paris, France) before every test session, as recommended by the manufacturer. The treadmill test provided an estimate of VO2max, defined as the highest oxygen consumption attained over a 15-second period during the testing protocol. To allow rapid comparison over a wide range of body weights (and especially with human data), dimensional analysis and empirical studies show that VO_2 should be expressed in relation to body mass raised to the power of 0,75 (Taylor et al. 1981; Mille-Hamard et al. 2012).

Familiarization

As previously described (Mille-Hamard et al. 2012), the mice were familiarized with the treadmill over a one week period *via* the completion of four 10min running sessions from 0 to 9 $m.min^{-1}$ (0, 3, 6 and 9 $m.min^{-1}$). All mice succeeded in running for the required time at an intensity of $9m.min^{-1}$. The velocity was not increased above this value, in order to avoid a training effect. The mice subsequently performed an incremental exercise test.

Incremental test load: VO2max determination

Starting from a speed of 10 $m.min^{-1}$, the exercise intensity was increased by $3m.min^{-1}$ every 2 min, with an incline of 15%. This protocol was used because it has been described as the test providing the higher VO_2max in mice (Høydal et al. 2007). Exercise continued until exhaustion, which was defined as an inability to maintain the running speed despite contact with the electric grid for more than 5 sec (Mille-Hamard et al. 2012). Exhaustion was then confirmed during the data analyses by the VO_2max stagnation while speed was still increased. All measurements were made by the same investigator. The last stage completed by the mouse was defined as the peak velocity (vPeak).

Exhaustive exercise

Exhaustive exercise was assessed using a modified protocol from Brooks & White (1978), at least 48 hours after maximal oxygen uptake determination to avoid interferences between the two tests (Mille-Hamard et al. 2012). Starting from a speed corresponding to 50% of vPeak, the exercise intensity was increased by 5% of vPeak every 2 min until reach 75% of vPeak, with an incline of 15%. At this moment, mice ran at this speed until exhaustion which was defined as an inability to maintain the running speed despite contact with the electric grid for more than 5 sec (Mille-Hamard et al. 2012). During this test, gas exchanges were not measured.

Animal were sacrificed directly at the end of exercise.

Hyperoxia exposure

Mice were housed in plexiglas chambers flushed continuously with oxygen from a liquid source (10 liter/min) and maintained at sea level atmospheric pressure by regulation of chamber outflow with a water manometer. This provided sufficient flow to maintain measured oxygen concentration consistently > 95% and CO2 concentration < 0.5%. Relative humidity was 50-70°. Lighting was on a 12-h on/off cycle. Food and water were provided ad libitum. Mice were observed every 6h for evidence of respiratory distress and survival. Survival time was recorded.

Blood and muscle sampling

Blood collection was done by a puncture to the abdominal aorta artery. Part of this blood was placed into a heparinized tube and was centrifuged at 1500g during 15 min at room temperature. Plasma was collected and stored at -20°C for later analysis. Other part of this blood was placed in a tube containing EDTA and then centrifuged at 1500g during 15 min at room temperature. It was then stored at -20 C for later determination of MDA.

Gastrocnemius muscle was collected and immediately frozen in liquid nitrogen.

Determination of glucose-6-phosphate dehydrogenase (G6PDH) activities in gastrocnemius muscle, liver and erythrocytes

Glucose-6-phosphate activity was determined using the techniques of Waller and co-workers (1986). Briefly, 1000 µl of glucose-6-phosphate (final []=10 mM) in potassium phosphate buffer was added to a cuvette. Then 400 µl of muscle or liver homogenate or erythrocyte lysate were introduced. The reaction was initiated by 400 µl of NADP (final []=0.90 mM) in buffer. The mixture was inverted and the absorbance read over 3 min at 340 nm using a spectrophotometer. Results were obtained in nmol x mg of protein-1 x min-1. Values were then normalized to those observed in the samples obtained from the WT group, which were assigned a value of 100%. Protein concentrations were determined by Bradford's method (Bradford 1976) by using bovine serum albumin as standard.

Damage determination

All the following measures were done in resting conditions and in response to exhaustive exercise kwon to induce oxidative stress (Ji 2001).

Determination of muscle damage

Muscle damage were evaluated by measuring lactate dehydrogenase (LDH) and creatine kinase (CK) plasmatic concentration. LDH and CK concentration assessment were carried into a Randox Daytona automate using commercial kits (Creatine Kinase RX series and lactate dehydrogenase RX series, RX Daytona^{TM}, Randox, France) according to the manufacturer's protocol.

Determination of systemic oxidative damage

Oxidative modification of total proteins in plasma were assessed by immunoblot detection of protein carbonyl groups using the 'OxyBlot' protein oxidation kit (Intergen) as previously described (Romagnoli et al. 2010). Approximately 20 mg of total protein was loaded onto gels and electrophoretically separated. Antibody anti-dinitrophenylhydrazone was purchased from Intergen Company (Purchase, NY). The procedure to quantify total protein carbonyls with the OxyBlot kit was densitometry of the blotting and of the Ponceau red staining (data not shown), followed by finding the ratio between the total density in the oxyblot and the total density in the Ponceau red staining. Specific proteins were visualised by using the enhanced chemiluminescence procedure as specified by the manufacturer

(Amersham). Autoradiographic signals were assessed using a BioRad scanning densitometer.

Lipids peroxidation determination as malondialdehyde (MDA) in plasma was performed by the method described by Wong et al. (1987). This method is based on the hydrolysis of lipid peroxides and subsequent formation of the adduct thiobarbituric acid (TBA) and MDA (TBA-MDA2). This adduct is detected by reverse phase HPLC and quantified at 532 nm (Ultimate 3000 Dionek). The chromatographic technique was performed in isocratic mobile phase being a mixture of 50 mM KH2PO4 (pH 6.8) and acetonitrile (70:30)

Determination of oxidative damage in gastrocnemius muscle

Oxidative modification of total proteins in gastrocnemius muscles was assessed by immunoblot detection of protein carbonyl groups using the "OxyBlot" protein oxidation kit (Millipore, Massachusetts) as previously described (Romagnoli et al. 2010). Approximately 20 mg of total protein was loaded onto gels and electrophoretically separated. Antibody anti-dinitrophenylhydrazone was purchased from Intergen Company (Purchase, NY). The procedure to quantify total protein carbonyls with the OxyBlot kit was densitometry of the blotting and of the Ponceau red staining (data not shown), followed by finding the ratio between the total density in the oxyblot and the total density in the Ponceau red staining. Specific proteins were visualised by using the enhanced chemiluminescence procedure as specified by the manufacturer (Amersham). Autoradiographic signals were assessed using a BioRad scanning densitometer.

Oxidative DNA damage was measured by 8-hydroxy-2′-deoxyguanosine (8-OHdG). A commercially available enzyme linked immunoassay (Highly Sensitive 8-OHdG Check, Japan Institute for the Control of Aging, Japan) was used to measure oxidized DNA in isolated muscle DNA samples. Gastrocnemius DNA was extracted *via* the High Pure PCR Template Preparation Kit (Roche, GmbH, Germany) according to the manufacturer's protocol. DNA was used if it had a minimum 260:280 ratio of 1.8. The assay was performed following the manufacturer's directions. Briefly, 50 µl of DNA were incubated with the primary antibody, washed, and then incubated in secondary antibody. The chromogen (3,3′,5,5′-tetramethylbenzidine) was added to each well, and incubated at room temperature in the dark for 15 min. The reaction was terminated and the samples were read at an absorbance of 450 nm. Samples were normalized to the DNA concentration measured *via* a plate spectrophotometer for nucleic acids (ND-2000, NanoDrop, Wilmington, DE). All analyses were done in triplicate.

Lipid peroxidation was evaluated by measuring 4 hydroxynonenal (4 HNE) modified protein using western blotting as described follow and specific anti-body (anti-4 hydroxynonenal abcam). It recognizes the specific group in the proteins that had been modified with HNE.

Antioxidant enzymes protein content was measured by western blotting as described follow.

Immunoblot analysis

Aliquots of gastrocnemius lysate (50-120 µg of proteins) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The whole gastrocnemius was used to ensure homogeneity. Proteins were then transferred to nitrocellulose membranes, which were incubated overnight at 4 °C with appropriate primary antibodies: anti-catalase (1:5000, Sigma Aldrich, Missouri); anti-G6PDH (1:1000, Abcam, UK); anti-Gpx (1:2000, Abcam, UK); anti-p38 (1:1000, Cell Signaling); anti-phosphorylated p38 (1:1000, Cell Signaling); anti-MuRF1 (1:200, Santa Cruz Biotechnology Inc, Santa Cruz, CA); anti-4 hydroxynonenal (1/500, Abcam, UK) and CuZnSOD (1:5000, NovusBio). Thereafter, membranes were incubated with a secondary antibody for 1 h at room temperature. Specific proteins were visualized by using the enhanced chemiluminescence procedure as specified by the manufacturer (Amersham Biosciences, Piscataway, NJ). Autoradiographic signals were assessed by using a scanning densitometer (BioRad, Hercules, CA). Data were represented as arbitrary units of immunostaining. To check for differences in loading and transfer efficiency across membranes, an antibody directed against α-actin (1:1000, Sigma Aldrich Missouri) was used to hybridize with all the membranes previously incubated with the respective antibodies. For the Western Blotting quantifications we first normalized all the proteins measured to α-actin. Samples from each group were run on the same gel.

Statistical Analysis

Statistical analyses were performed using the SigmaStat 3.1 Program (Jandel Corp., San Rafael, CA). Results are expressed as mean ± SD. Normality of distribution was checked with the Kolmogorov test and homogeneity of variance was tested by Levene's statistics. We used a student-test to compare group differences in resting conditions. Differences were considered significant if $p<0.05$. To test for statistically significant differences between the groups in the pro-oxidizing situations a two-way ANOVA was used. When significant F-ratios were observed, a Bonferroni multiple comparison's test was applied to test individual means. Statistical significance was assumed at $p<0,05$.

RESULTS

G6PDH overexpression increases G6PDH activity and protein content

To confirm that G6PDH overexpression was effective, we measured G6PDH activity in various tissues and protein content especially in skeletal muscle because it was the tissue of interest in this work. Results are shown in Figure 2. G6PDH overexpression multiplied G6PDH activity by 1,5 in erythrocytes (see panel A, $p<0,01$), by 2 in gastrocnemius muscle (see panel B, $p<0,01$) and by 4,5 in liver (see panel C, $p<0,01$) in G6PDHtg mice compared to WT mice. G6PDH muscle protein content was twofold higher in G6PDHtg mice compared to WT mice (see panel D, $p<0,01$).

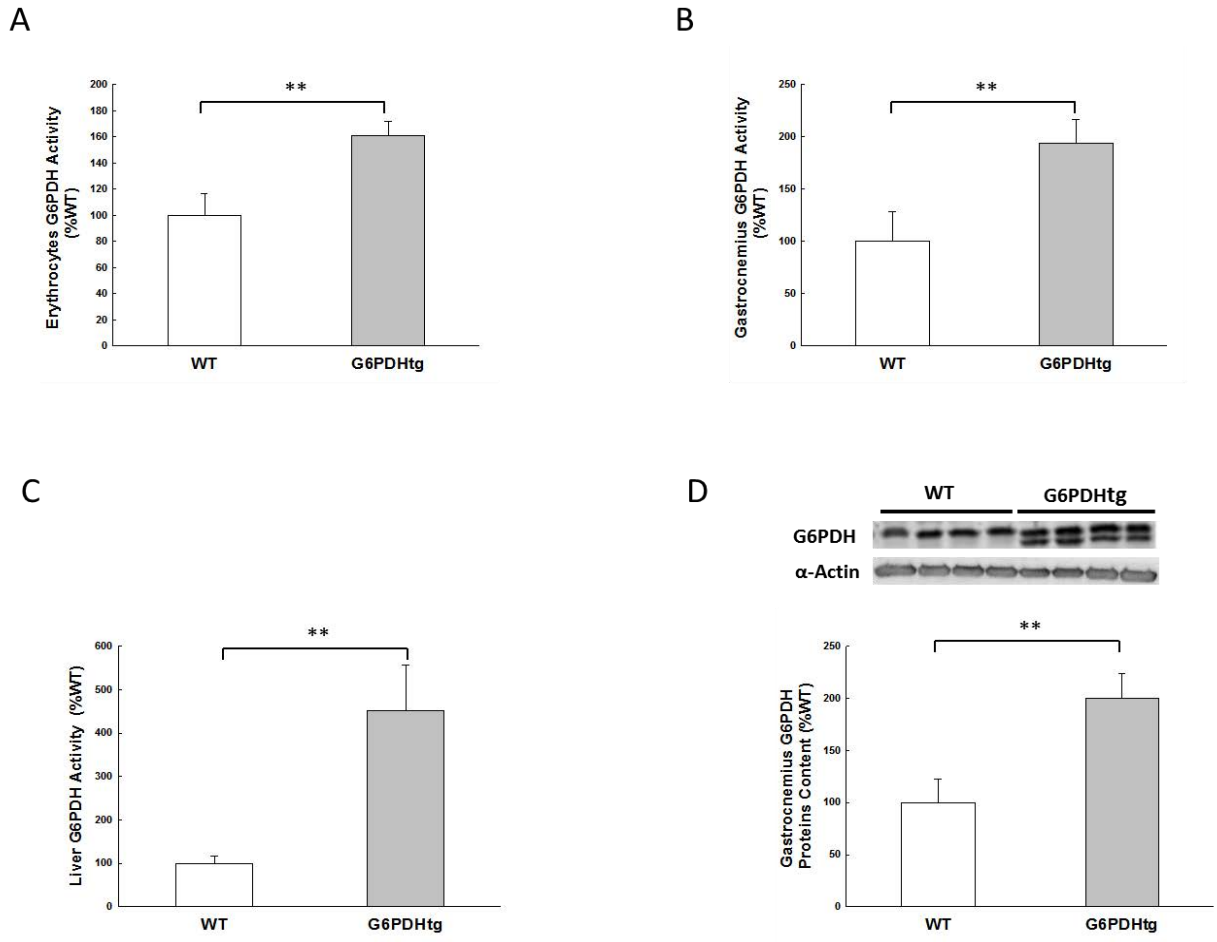


Figure 2. G6PDH overexpression increases G6PDH activity and protein content.

Animals were divided into two experimental groups: Wild type (WT) (n=8) and G6PDH transgenic mice (G6PDHtg) (n=8). Panels A, B and C show G6PDH enzymatic activity respectively in erythrocytes, gastrocnemius muscle and liver. Western blotting analysis to detect G6PDH (Panel D) in mice gastrocnemius muscle was performed. Representative blots

are shown. The content of α-actin, a housekeeping protein marker in skeletal muscle, was determined in all the experimental groups. Values were normalized to those observed in the samples obtained from the WT group, which was assigned a value of 100%. Values are shown as mean (±SD). **p<0,01.

Oxidative stress in resting conditions

G6PDH overexpression does not modify systemic oxidative damage but decreases gastrocnemius oxidative damage

Numerous studies in rodents have shown that decreased G6PDH activity and protein content is associated with increased oxidative damage (Kumaran et al. 2004; Senthil Kumaran et al. 2008; Braga et al. 2008; Kovacheva et al. 2010). In order to assess if G6PDH overexpression could protect against oxidative stress (OS), we measured in resting conditions oxidative damage in plasma (which are used to assess systemic OS) and gastrocnemius muscle. Our results showed that neither carbonylated protein and malondialdehyde level were altered by G6PDH overexpression (see figure 3, Panels A and B).

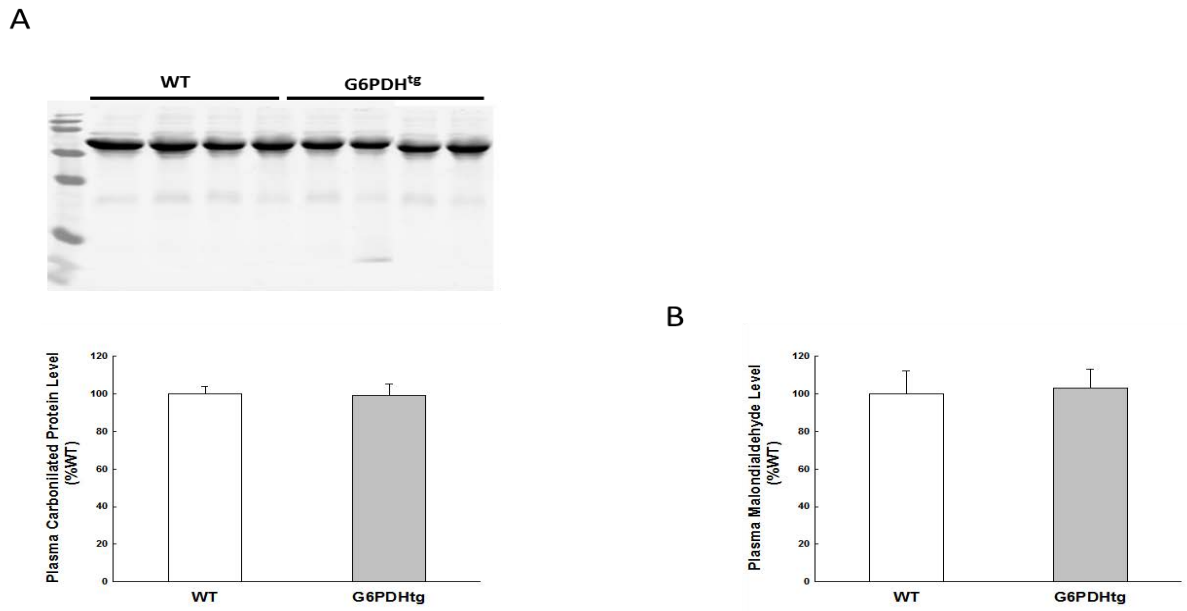


Figure 3. G6PDH overexpression does not improve systemic oxidative damage in resting conditions.

Animals were divided into two experimental groups: Wild type (WT) (n=8) and G6PDH transgenic mice (G6PDHtg) (n=8). Panel A shows a Western blotting analysis to detect protein carbonylation in plasma. A representative blot is shown. For the densitometric

analysis of the results, values are shown as mean (±SD). Panel B presents plasmatic malondialdehyde level. Values were normalized to those observed in the samples obtained from the WT group, which was assigned a value of 100%. Values are shown as mean (±SD).

In gastrocnemius, carbonilated proteins and 4 hydroxynonenal modified proteins were similar between G6PDHtg and WT mice (see figure 4, panels A and B). Panel C represents 8-hydroxy-2′-deoxyguanosine (8-OHdG) gastrocnemius content. Here, we observed a significantly 23% lower levels of 8-OHdG gastrocnemius content in G6PDHtg compared to WT mice (**p<0,01).

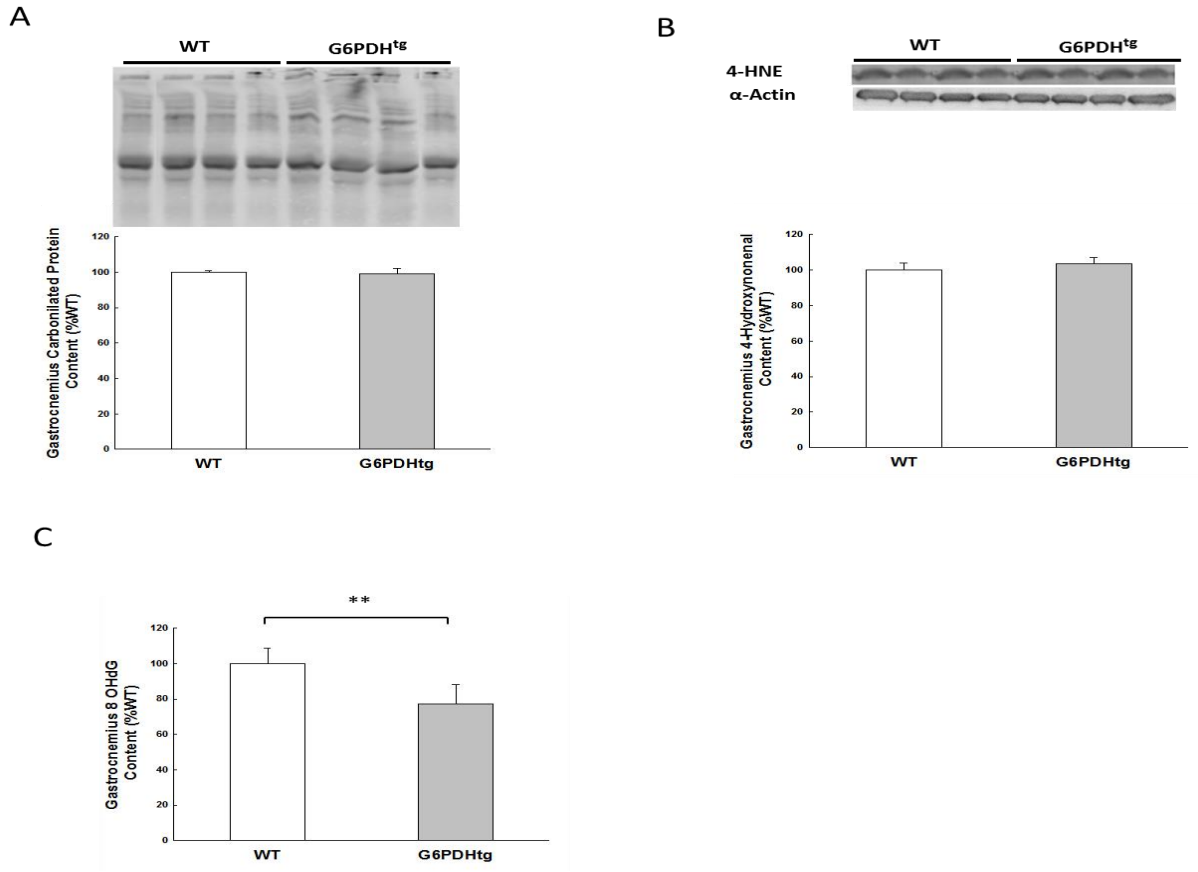


Figure 4. G6PDH overexpression improves gastrocnemius oxidative damage in resting conditions.

Animals were divided into two experimental groups: Wild type (WT) (n=8) and G6PDH transgenic mice (G6PDHtg) (n=8). Panel A and Panel B show respectively a Western blotting analysis to detect protein carbonylation and lipids peroxidation in gastrocnemius. A representative blot is shown. For the densitometric analysis of the results, values are shown as mean (±SD). Panel C shows 8-OHdG from DNA extracted from gastrocnemius muscle of

mice. Values were normalized to those observed in the samples obtained from the WT group, which was assigned a value of 100%. Values are shown as mean (±SD) **p<0,01.

Overexpression of G6PDH is not associated with increased antioxidant enzymes in gastrocnemius

Several antioxidant systems depend on the production of NADPH by G6PDH for proper function such as the glutathione system (M. D. Scott et al. 1993), catalase (M. D. Scott et al. 1993) and indirectly the superoxide dismutase (SOD) (Stanton 2012). In order to assess if G6PDH overexpression led to improve antioxidant system, we analyzed by western blotting the protein expression of the aforementioned systems. Figure 5 resumes our results. We did not found any difference between G6PDHtg and WT mice in the protein content of glutathione peroxidase-1 (Gpx-1), CuZn superoxide dismutase (CuZnSOD) and catalase in gastrocnemius.

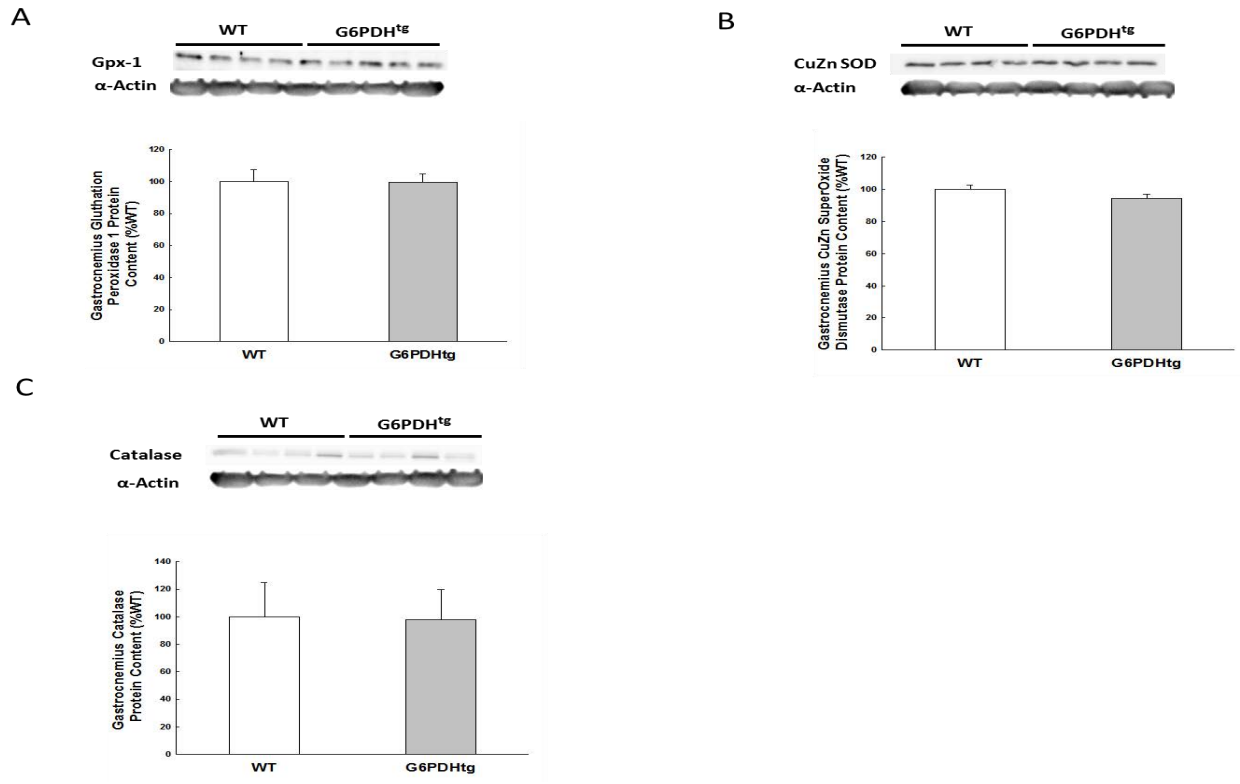


Figure 5. G6PDH overexpression does not improve gastrocnemius antioxidant enzymes in resting conditions.

Animals were divided into two experimental groups: Wild type (WT) (n=8) and G6PDH transgenic mice (G6PDHtg) (n=8). Panel A, B and C show respectively a Western blotting

analysis to detect glutathione peroxidase-1 (Gpx-1), CuZn superoxide dismutase (CuZnSOD) and catalase in gastrocnemius. A representative blot is shown. For the densitometric analysis of the results, values are shown as mean (±SD). Values were normalized to those observed in the samples obtained from the WT group, which was assigned a value of 100%. Values are shown as mean (±SD).

Survival assay in hyperoxia

Based on the study of Legan et al. (2008) in which flyies overexpressing G6PDH were protected against death induced by hyperoxia (situation known to produce a very intensive oxidative stress), we decided to repeat the experiment in our model. In order to determine the effects of G6PD overexpression on resistance to oxidative stress, mice were exposed continuously to hyperoxia ($[O_2] > 95\%$, $[CO_2] < 0.5\%$). The maximal and survival time between G6PDHtg and WT mice was similar (around 90 hours; data not shown). Note that this experience was a pre-experiment carried on only 4 animals per group for ethical reasons. Based on the lack of difference between G6PDHtg and WT mice and for ethical reasons, the experiment was not repeated with more animals.

Oxidative stress in response to exhaustive exercise

As mentioned in study 2, G6PDH overexpression is associated with an increased maximal oxygen uptake (data not shown).

The diagnosis of muscular lesions in response to exercise is usually made by blood tests to detect the presence of specific muscle markers (Guerrero et al. 2008). Creatine kinase (CK) and lactate dehydrogenase (LDH) are among the most widely used plasma markers of muscle damage. To assess if G6PDH overexpression was able to protect against muscle damage in response to exercise, plasmatic CK and LDH were measured. Results are presented in figure 6 (respectively panel A and B). For both CK and LDH, there were no differences in the control (= non exercise) groups (WTC: WT control and TGC: G6PDH control). Independently of the genotype, we observed a significantly increase in CK and LDH after exercise in the WTE and TGE groups which has the same amplitude (CK: WTE *vs* WTC = +70%, TGE *vs* TGC = +75%; LDH: WTE *vs* WTC = + 116%, TGE *vs* TGC = + 129% ; $p<0,05$ in all cases). It has been previously found that free radicals are, at least in part, responsible of the muscle damage in response to exercise (Gómez-Cabrera et al. 2003). As

previously described, G6PDH plays an important role in the antioxidant systems. Thus, to assess if G6PDH overexpression protects against muscle oxidative damage induced by exercise, carbonilated proteins and 4 hydroxynonenal modified proteins (4-HNE) were measured. Results are shown figure 6 (respectively panel C and D). For both carbonilated proteins and 4 HNE, there were no differences in the control groups (WTC and TGC). Independently of the genotype, we observed a significantly increase in carbonilated proteins and 4-HNE in the WTE and TGE groups which has the same amplitude (carbonilated proteins: WTE *vs* WTC = +22%, TGE *vs* TGC = +29% ; 4-HNE: WTE *vs* WTC = + 35%, TGE *vs* TGC = + 33%; $p<0,05$ in all cases).

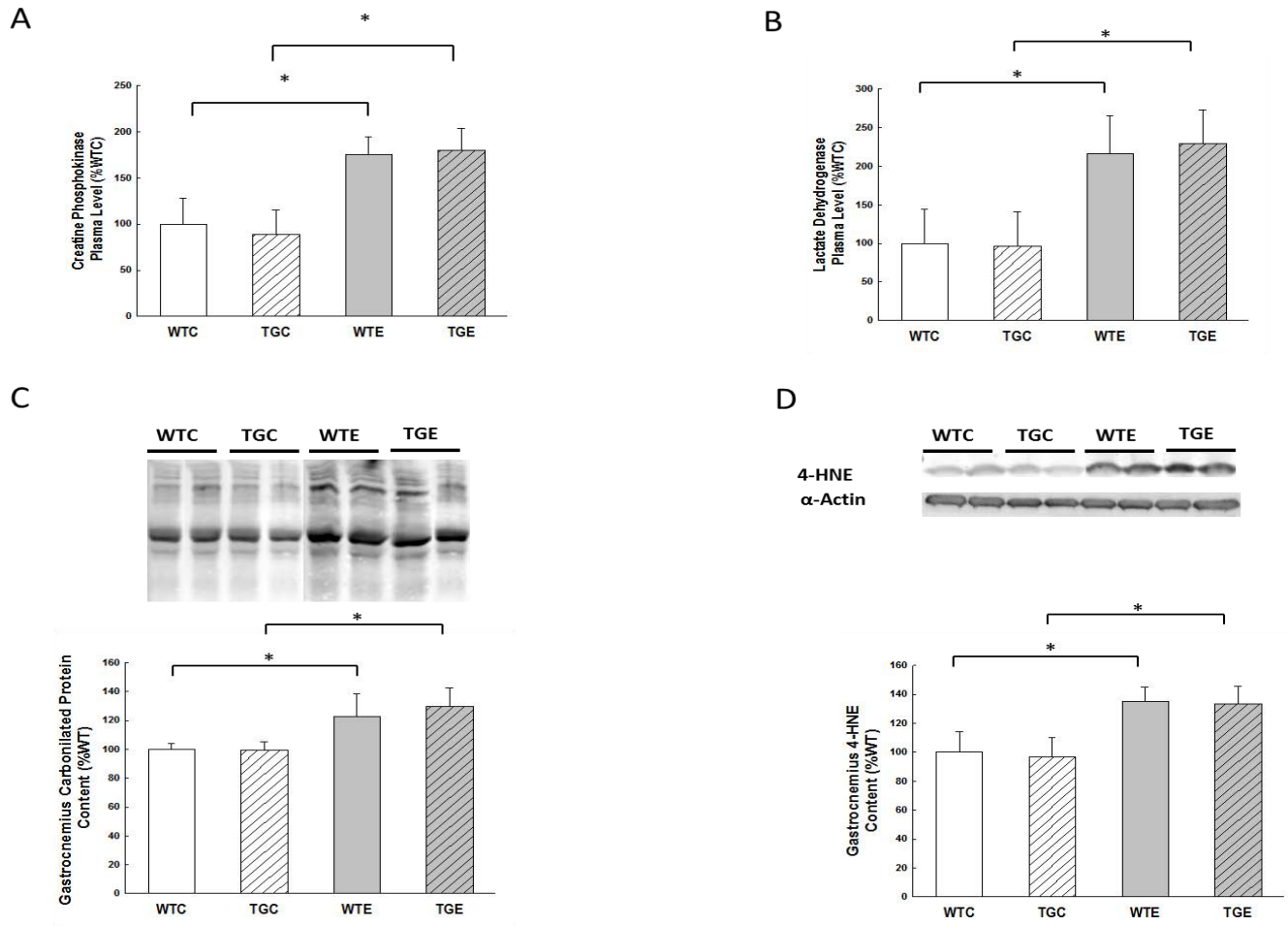


Figure 6. G6PDH overexpression does not protect against exercise-induced muscle damage

Animals were divided into four experimental groups: Wild type control (WTC) (n=5), G6PDHtg control (TGC) (n=5), WT exercise (WTE) (n=5) and G6PDHtg exercise (TGE).

Panel C and D show respectively a western blotting analysis to detect protein carbonylation and lipids peroxidation in gastrocnemius. A representative blot is shown. For the densitometric analysis of the results, values are shown as mean (±SD). Values were normalized to those observed in the samples obtained from the WT group, which was assigned a value of 100%. Values are shown as mean (±SD). *$p<0,05$.

Discussion

The present study was conducted to verify whether overexpression of G6PDH have a beneficial impact on oxidative stress in resting conditions and in response to pro-oxidizing stimuli. Our data clearly showed only a decrease in DNA oxidative damage in G6PDHtg mice skeletal muscle in resting conditions. Based on the markers studied, we did not find a protective effect of G6PDH overexpression in response to oxidative stress induced by pro-oxidizing stimuli.

As in our previous study, we confirmed that G6PDH overexpression was effective in the G6PDHtg mice by measuring its activity and protein expression in several tissues and notably in skeletal muscle where the activity and the protein content were two fold higher compared to WT mice.

Concerning the resting conditions, our results revealed that protein oxidation (evaluated through carbonilated proteins) and lipid peroxidation (evaluated *via* malondialdehyde) were not reduced by G6PDH overexpression. This lack of difference between G6PDHtg and WT mice reinforces indirectly data concerning these same markers in G6PDH human deficient patients. Indeed, with the exception of Nikolaidis et al. (2006), Jamurtas et al. (2006) and Theodorou et al. (2010) who reported that lipid peroxidation (assessed by lipid hydroperoxydes and Thiobarbituric acid reactive substances) and protein oxidation (evaluated by carbonilated proteins) were similar in G6PDH deficient humans compared to their healthy counterpart, no study has yet investigated these effects. Indeed, unfortunately, in our knowledge no data on the animal systemic oxidant stress exist either in response to a G6PD deficiency or overexpression. Moreover, the only team (Corcoran et al. 1996) that has been working in our model did not explored these systemic parameters. Our study is the first that has focused on the role of G6PDH on systemic oxidative stress in animals.

Protein oxidation (evaluated through carbonilated proteins) and lipid peroxidation (evaluated *via* 4-HNE) were similar in G6PDHtg mice compared to WT mice supposing that muscle oxidative damage appear to be independent of G6PDH status. However, controversial findings as an increased oxidative damage in specific tissues associated to a decreased G6PDH activity and or protein content have been observed. Thus, Kumaran et al. (2004, 2008) and Kovacheva et al. (2010) showed in aged rodents that a decrease in G6PDH activity or protein content was associated with an increase in lipid peroxidation (measured as 4-HNE and lipid hydroperoydes) and a decrease in GGSH/GSSG ratio. These divergences could be

explained by the age of our animals. Compared to the studies of Kumaran et al. (2004, 2008) and Kovacheva et al. (2010, our mice were young and at this age, basal oxidative stress is well controlled by the antioxidant systems, at least in skeletal muscle. Indeed, the aformenionned studies were carried in senescent animals aged between 18 and 22 months old while we were working in adult animals aged between 12 and 14 months old.

For the first time, our study also revealed that G6PDH overexpression led to a decrease in total DNA oxidative damage in gastrocnemius. Indeed, 8-OHdG values were significanlty lower in the G6PDHtg mice compared to the WT mice. Although this relation has never been stutied in skeletal muscle, others found concordant results in brain. Thus, Felix et al. (2002) and Jeng et al. (2013) found higher 8-OHdG values and others DNA damage markers in the brain of G6PDH deficient mice compared to their WT conterpart. In both case, total DNA damage were associated with increased DNA mutations. The mechanism by which G6PDH would act on DNA is not clear and need to be studied. As previously exposed, brain DNA damage led to brain DNA mutations (Felix et al. 2002; Jeng et al. 2013). Although this relation was not researched in our study, we can suppose that our mice with lower muscle DNA damage would have also lower muscle DNA mutations. This data is very essential since numerous studies observed in various species an age-related accumulation of mitochondrial DNA mutations in skeletal muscle induced by the oxidative stress (Lee et al. 1998; Bua et al. 2006; Figueiredo et al. 2009; Lee et al. 2010). These mitochondrial DNA mutations led to electron transport chain abnormalities associated with morphological aberrations of muscle fibers (Bua et al. 2006). Thus, since it has been shown that G6PDH is present in mitochondria (Mailloux & Harper 2010), we would suppose that our G6PDHtg mice with potential lower mitochondrial DNA mutation would be protected during aging.

Our G6PDHtg mice were not protected against hyperoxia. Indeed, the survival time during hyperoxia exposure was the same between the G6PDHtg mice and their WT counterpart. In the same way, we did not find any protective effect of G6PDH overexpression against muscle damage and muscle oxidative damage induced by exhaustive exercise. Indeed, we found similar increases of plasmatic CK and LDH concentrations as well as similar increases in carbonilated protein and 4-HNE modified protein in G6PDHtg and WT mice. The absence of a protective effect against induced oxidative stress could be explained by the fact that G6PDH does not supply NADPH only to antioxidant systems. Indeed, if G6PDH is known to supply NADPH to the glutathione system and catalase (M. Scott et al. 1993), it has been reviewed that G6PDH supplies NADPH to xanthine oxidase, nitric oxide synthase and NADPH oxidase (Hecker & Leopold 2013). All these enzymes have been shown or are

supposed to be involved in free radical production during exercise and notably exhaustive exercise (Gomez-Cabrera et al. 2005; Gomez-Cabrera et al. 2010; Gomez-Cabrera et al. 2013). Consequently, the increased antioxidant capacity that G6PDH would bring by supplying NADPH to antioxidant systems would counterbalanced by the NADPH supply to pro-oxidant systems. However, we did not measure the activity of these pro-oxidant enzymes. The same reasons would explain the lack of difference observed during hyperoxia.

In summary, we found that G6PDH overexpression in mice clearly decreased DNA oxidative damage in skeletal. This result reinforces the role played by the G6PDH in the DNA protection since deficient G6PDH mice presented higher DNA oxidative damage. However, the mechanisms by which this protection is effective involved the glutathione but the exact mechanism have to be studied. Surprisingly, the expected protective effect of G6PDH overexpression against oxidative stress induced by pro-oxidizing stimuli was not present surely due to compensatory mechanisms between the antioxidant systems and pro-oxidant systems depending of G6PDH for the NADPH supply.

GENERAL DISCUSSION

DISCUSSION

Age-induced chronic oxidative stress in skeletal muscle is associated with altered cell signaling pathways leading to sarcopenia.

Our study provided new evidences *in vivo,* that impairment of muscle cell signaling pathways involved in sarcopenia are induced by a chronic oxidative stress. Indeed, we clearly showed that aging was associated with a chronic oxidative stress in skeletal muscle since oxidative damage (proteins and DNA oxidation) in our old rats were higher compared to our young animals. Our study confirmed previous results published by Radák et al. (2002) and Jackson et al. (2011) which have also shown an increase in DNA oxidation and lipids peroxidation in older rodents. Our evaluation of oxidative damage could have been completed by markers of lipid peroxidation such as malondialdehyde or isoprostanes, also described to increase during aging in skeletal muscle (Kovacheva et al. 2010). However, proteins and DNA oxidation, evaluated in our study, appear to be more relevant than lipids peroxidation due to their implications in regulating muscle mass. The increase of oxidative damage is attributed to the decrease of antioxidant systems efficiency since we showed a lower protein expression of catalase, glutathione peroxidase and G6PDH which are major antioxidant enzymes (M. D. Scott et al. 1993). Our data were in accordance with previous authors who reported, in aged rodent a decrease in antioxidant enzymes protein content in skeletal muscle (Senthil Kumaran et al. 2008; Braga et al. 2008; Kovacheva et al. 2010). However, such results have not been always found. Indeed, Ryan et al. (2008) did not find any change in antioxidant enzyme such as Gpx and catalase. Otherwise, several studies reported an increase in antioxydant enzymes activity in skeletal muscle during aging in rodent (Ji et al. 1990; Ryan et al. 2008). In our study, activity of antioxidant enzyme activity was not performed. However, if like in the aforementioned studies, the activity of the antioxidant enzyme was increased, the higher oxidative damage observed in our old rats compared to the young would witness their inability to counteract the age-induced oxidative stress.

Thereafter, we have shown that this oxidative stress was associated with an impairment of the PI3K/Akt/mTOR pathways since we observed a decreased Akt and p70S6K activation. Our results suggest that aging was associated with an impaired protein synthesis as found in others studies in older rodents (Haddad et al. 2006; Thompson et al. 2006). The relation between oxidative stress and impairment of the PI3K/Akt/mTOR have been already shown *in vitro* (Shenton et al. 2006; O'Loghlen et al. 2006; Zhang et al. 2009), and our study brings a prove that it could really occur *in vivo*. This potential decreased protein

synthesis is important since it would participate in the muscle atrophy associated to sarcopenia which could lead to impaired muscle strength as found in others studies (Chabi et al. 2008; Andersson et al. 2011). As muscle mass is controlled by a balance between protein synthesis and proteolysis (Powers et al. 2012), we evaluated several markers of the ubiquitine-proteasome system (UPS) in regard to the increased oxidative damage that we found. Our results showed an increase in the muscle protein content of MuRF1 and MaFbx, the most two important ubiquitin ligases involved in muscle proteolysis (Foletta et al. 2011). Our results are concordant with the results of others studies that have already found such results (Hepple et al. 2008; Altun et al. 2010). Moreover, they showed that increased MuRF1 protein content was associated with an increased proteasome activity. However, these studies did not focus on the relation between oxidative stress and UPS. Based on our results and these published by Altun et al. (2010), we suggested that our old rats were presenting a higher proteolysis compared to their young counterpart. However, such results have not been always found. Indeed, studies found that MAFbx and MuRF1 expression can be unchanged during aging in skeletal muscle (Léger et al. 2008; Whitman et al. 2005). To confirm, an increased proteolysis through the UPS, it would be interested to directly measure the proteasome-dependent proteolysis. On the other hand, our results would confirm data obtained *in vitro* showing that exposure of C2C12 myotubes to H_2O_2 (known to increase during sarcopenia) up-regulated the expression of MuRF1 and Atrogin-1 (Li et al. 2003).The impairment of the PI3K/Akt/mTOR pathway as well as the up-regulation of UPS compounds, were reinforced by the increased myostatin protein expression observed in our aged rats. Noted that myostatin is known to up-regulate the UPS and inhibit the PI3K/Akt/mTOR pathway (Amirouche et al. 2009; Sartori et al. 2009; Trendelenburg et al. 2009). Our data supposed a link between oxidative stress and myostatin that would be interested to study. Our results are also in accordance with Ploquin et al. (2012) who indicated that absence of myostatin in mice is associated with lower oxidative damage and increased antioxidant enzyme.

Associated with the oxidative damage, our older rats are characterized by a decrease in mitochondrial content and activity. These data which confirm those of Lee et al. (2010) and O'Leary et al. (2013) could be explained by the age-increased ROS production by the mitochondria which leads to an accumulation of oxidative damage to the mitochondrial compounds especially to mtDNA leading to mtDNA mutations. These mutations induce the synthesis of defective ETC subunits leading to an impaired mitochondrial function (Harman 1972; Miquel et al. 1980). Moreover, we also found a decreased activation of the mitochondriogenesis pathway (i.e. PGC-1α, NRF-1) as previously reported by numerous

studies (Chabi et al. 2008; Derbré et al. 2012; Koltai et al. 2012; Ibebunjo et al. 2013; Pugh et al. 2013). Mitochondria content and activity are a key component in the maximal oxygen consumption. Consequently, a decrease in their content and activity would lead to decrease the aerobic qualities of elderly people as it has been reported by Capel et al. (2005)

An impaired muscle regeneration capacity is often supposed to occurring during aging due to an pro-oxidizing cellular environment (Carlson & Faulkner 1989; Carlson, Suetta, et al. 2009). In our study, we observed a down-regulation of Myf-5 which is a well-known marker of myoblast/satellite cell differentiation (Kim et al. 2005) and an up-regulation of p21 which is a cell cycle inhibitor (Jaumot et al. 1997). These results in addition the concomitant myostatin increase that we observed suggested a possible impairment of muscle regeneration in our older rats. Indeed, myostatin is known to maintain satellite cell quiescence status and repress cell-renewal through the induction of p21 (McCroskery et al. 2003), suggested a possible impairment of muscle regeneration in aged muscle. However, additional measures would be needed to confirm this hypothesis.

After having confirmed, that age-related oxidative stress was associated with impairment of several pathways involved in sarcopenia, we showed that oxidative stress was associated with muscle atrophy. Indeed, the higher specific gravity index (showing a decreased lean mass and an increased adiposity) and the lower muscle weight/body weight ratio (attesting the muscle atrophy) observed in our old rats allowed us to qualify them as sarcopenic. Our results agreed with other studies showing that at the same age (22-24months) rats presented muscle atrophy associated with a chronic oxidative stress (Capel et al. 2004; Mosoni et al. 2004).

Growth hormone replacement therapy is effective to combat sarcopenia by improving protein synthesis and mitochondriogensis associated with restoring a young redox profile

Growth hormone can be used in two ways in hormone therapies during aging. GH can be given to raise its concentration above the normal values found in younger people or to raise similar values. Since high doses of GH cause high incidence of adverse effects (Papadakis et al. 1996; Holloway et al. 1994), we decided to use a dose allowing to reach similar plasmatic IGF-1 levels than those found in younger rats. The dose and duration were chosen based on previous studies published by the team of Professor Tresguerres which has a long experience in GH replacement therapy in older rodents (Castillo et al. 2004; C Castillo et al. 2005;

Carmen Castillo et al. 2005; Kireev et al. 2007). In order to simulate a pulsatile secretion which is a main drawback reported in the human trials (Giannoulis et al. 2012), GH was given in two daily doses at 10h and 17h.

A main finding of our study is that GH replacement therapy was useful in preventing the age-related muscle mass loss. Indeed, our data showed that GH administration significantly increased specific gravity index in old male rats, which means that GH, through its anabolic, antilipogenic and lipolytic properties, is able to increase muscle mass and reduce body fat (Castillo et al. 2004; Carmen Castillo et al. 2005). Moreover, we found that the gastrocnemius muscle atrophy observed in old non-treated rats was significantly prevented in the old treated ones. These beneficial effects were surely driven by an increased protein synthesis as supposed by the increased activation of Akt and p70S6K and a decreased proteolysis as supposed by the decreased MuRF1 expression. The down-regulation of myostatin observed in our old treated rats could explain these results. Indeed, in the absence of myostatin, mice showed an increased protein synthesis due to an increased activation of the PI3K/AkT/mTOR pathway (Guo et al. 2009). Moreover, inhibition of myostatin with specific inhibitor has been shown to decrease MuRF1 expression in the skeletal muscle of mice (LeBrasseur et al. 2009). We were the first to report that GH hormone treatment is able to down-regulate myostatin. Data has been previously found in transgenic salmon overexpressing growth hormone exhibiting decreased myostatin transcript and protein expression (Roberts et al. 2004). This interesting result need to be confirmed in further investigations.

Interestingly, the GH treatment used in our study led to improve mitochondriogenesis in the treated rats. Recently, Vescovo et al. (2005) working in cardiac muscle in rats, reported that GH activates PGC-1α *via* IGF-1 and calcineurin. It would be interested to explore this pathway in our study. In the same way, Short et al. (2008) demonstrated in healthy young humans that acute GH action promotes an increase in mitochondrial oxidative capacity and abundance of several mitochondrial genes (e.g. COX3, TFAM). However, mitochondrial protein synthesis was not increased surely due to the unique dose of GH. An increase in mitochondriogenesis is usually associated with an increased maximal oxygen consumption (Short et al. 2003; Koltai et al. 2012) and/or an increased endurance capacity (Derbré et al. 2012). Such effect in sarcopenic elderly would improve their health span.

Interestingly, the restoration of a normal activation of the PI3K/AkT/mTOR pathway and mitochondriogenesis, as the decreased expression of MURF1 were concomitant to an improvement of the redox status. Indeed, GH treatment decreased protein and DNA oxidation

in our old treated rats to values similar to those observed in the young rats. The observed up-regulation of the expression of important intracellular antioxidant enzymes, such as catalase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase in the old treated rats would explain this result. However, the exactly mechanism and the actor by which GH activated the expression of antioxidant enzymes is still unknown. A decrease in the age-related oxidative damage has been also reported in response to another hormone replacement therapy by Kovacheva et al. (2010). Indeed, in response to testosterone treatment in old mice, they showed a decrease in lipid peroxidation associated with an increased Akt activation. According to these authors, these modifications will be associated with an increased G6PDH protein content. These similar results suggested that growth factors in general are able to improve oxidative stress. Since our study demonstrated also an increase in the G6PDH protein content, we can suppose that the beneficial of our GH treatment would involve the supply of NADPH to the glutathione system and catalase (M. D. Scott et al. 1993). Consequently the capacity to scavenge free radical would be increased and would explain the decrease in oxidative damage. However, these hypotheses have to be confirmed.

Glucose-6-phosphate dehydrogenase overexpression, body composition, physical performance and oxidative stress: discussion around developing strategies in order to combat sarcopenia.

The aim of our second study, was to study the roles of the G6PDH enzyme in regulating body composition (muscle mass and fat mass) and its impacts on physical performances (muscle strength, maximal oxygen uptake and endurance capacity). The main finding of the study was that overexpression of G6PDH in mice improved body composition by decreasing fat mass and increasing muscle mass surely due to an increase in the protein synthesis capacity through an increase in muscle DNA content. Improvement in body composition was associated with better muscle strength and aerobic qualities.

The aim of the third study was to study if the overexpression of G6PDH has a beneficial impact on oxidative stress in resting conditions and in response to pro-oxidizing stimuli. Our data clearly showed a decrease in DNA oxidative damage in G6PDHtg mice skeletal muscle in resting conditions and surprisingly we did not find a protective effect in response to pro-oxidizing stimuli. However, we also did not find adverse effect in response of this overexpression.

Since sarcopenia is characterized by a loss of muscle mass, strength, physical performance (Cruz-Jentoft et al. 2010) and also increased fat mass (Visser et al. 2005), our aforementioned results provide evidence that developing strategies which would lead to up-regulate G6PDH would potentially effective to combat sarcopenia.

The fact that G6PDH overexpression leads to increase muscle mass brings for the first time the proof that this enzyme is effectively involved in the regulation of muscle mass as it suggested by several studies (Wagner et al. 1978; Max 1984; Kovacheva et al. 2010). However, our results do not allow to determine the exactly role of the G6PDH in regulating muscle mass. Based on our results showing an increased muscle DNA content, we suppose that this latter enhances the protein synthesis of the G6PDHtg mice, since from a theoretical point of view DNA content is the limiting factor of transcription capacity. Increase in protein synthesis would lead to a higher muscle mass. *In vitro* data support this idea. Indeed, Tian et al. (1998) showed that cells overexpressing G6PDH were growing faster than WT cells due to an increase DNA synthesis. Moreover, this increased DNA synthesis was associated with an increased protein synthesis (Tian et al. 1998). In our study, we measured the activation of Akt and P70SK6 which have been found to increase protein synthesis when activated in skeletal muscle (Kimball et al. 2002). However, we did not find any change when compared to the WT type mice. However, based only on these two markers, it is not possible to conclude if the protein synthesis was really unchanged. Measuring others markers such as 4EBP1 and rpS6 or measuring directly protein would complete this analysis. Although, the mechanisms by which G6PDH overexpression increases muscle mass are still under investigation in our laboratories, our results provide new insight to develop strategies to fight against sarcopenia or in more large field to gain muscle mass. Moreover, thanks to the results found in our third study, we demonstrated that having more G6PDH is not deleterious in pro-oxidizing condition such as exercise. Indeed, based on our results, in response to a pro-oxidizing stimulus, the lack of difference between G6PDHtg and WT mice suggest that the antioxidant and the pro-oxidant systems which depend of G6PDH through the NADPH supply reached an equilibrium. Consequently, use strategies which would up-regulate G6PDH would not have deleterious effect on oxidative stress. However, we need before studying the repetition of the exposition to pro-oxidizing stimuli.

Associated to this increased muscle mass, the G6PDHtg mice showed a decrease in fat mass which was very surprising since an increase in G6PDH activity have been usually associated with a adipose tissue lipogenesis due to higher G6PDH activity in this organ (Park et al. 2005b; Bonnet et al. 2007; Zomeño et al. 2010). Indeed, it has been shown that G6PDH

deficient mice are protected against weight gain in response to obesogenic food (Hecker et al. 2012). Based on this study, our G6PDHtg mice would be more exposed to weight gain in response to the same protocol. However, Bonnet et al. (2007) indicated in cattle that the lower rib fat thickness was observed in strain with higher mRNA levels and activities of G6PDH. All these contradictory effects underline the need to understand the exact role played by G6PDH in the lipid metabolism.

The higher maximal oxygen consumption and better results in the different strength tests found in the G6PDHtg mice compared to the WT mice supply a news arguments to develop strategies targeting G6PDH. However, the better results obtained may be principally due to the lower weight observed in the G6PDHtg since all the values of the different tests were normalized to the body weight as preconized. Normalized, these values by the lean mass would allow differentiating the biological effect from the weight effect. Mice who realized the originally DXA could not be used in this way, since they did not realize the different tests to explore physical performance. However, the mice whose has performed these tests have been evaluated with bio-impedancemetry, and the data are still under-validation.

Although, we present arguments in favor to develop strategies targeting G6PDH to gain muscle mass, lose fat mass or improve physical performance, some populations in particular those presenting a high risk of cancer or those with cachexia would be excluded. Indeed, it has been underlined a possible oncogenic role of G6PDH which would worse the risk of cancer (Kuo et al. 2000; Zhang et al. 2013). In part for this reason, GH is not currently used in elderly people.

Due to the fact that G6PDH only protects the DNA from oxidative damage, we could ask if this protection is really due to an antioxidant mechanism. Indeed, in regards to our results, a different mechanism could be hypothesized. Thus, the higher DNA content and the higher plasma uric acid levels revealed a more activated nucleotides turnover. We cannot exclude that it will lead to a more frequent renewal of the nucleotides composing the DNA. The latter would then be less exposed to free radicals and consequently the DNA would present less oxidative damage.

Finally, based on our contradictory effects, we can ask if use this model is the best approach to study the role of G6PDH in skeletal muscle as we did.

In order to confirm the highlighted role of G6PDH in regulating muscle mass, doing a transgenic which would overexpress G6PDH only in skeletal muscle could be a better approach. On the other hand, an *in vitro* approach, using satellites cells extracted from these mice could be envisaged.

CONCLUSION

The life expectancy has never been so long in the history of humankind. However, it leads to the aging of the general population and inevitably to an increase in the prevalence of sarcopenia, which in turn increase dramatically the healthcare costs of our societies. In order to limit this phenomenon, to develop effective strategies to prevent or treat sarcopenia is a major challenge that requires understanding the cellular and molecular involved in its onset and those leading to its prevention.

This thesis attempted to answer three general objectives. The first objective was to determine *in vivo* to what extent a pro-oxidant redox status within the aged muscle tissue may modulate signaling pathways involved in cellular mechanisms underlying sarcopenia. The second objective was to show that return to normal functioning of these signaling pathways requires a restoration of the redox homeostasis. Finally, the third objective of this thesis was to identify actors and their possible cellular mechanisms in the maintenance and/or the restoration of the redox status.

In a first study realized in old rats, we found that age-related oxidative stress leads to an impairment of the PI3K/AkT/mTOR pathway suggesting a decrease in protein synthesis while in the same time an increase in the expression of MuRF1 and Mafbx suggested an increase in proteolysis through the ubiquitine-proteasome system. Moreover, a decreased mitochondrial function and genesis was found.

In a second time, we found that growth hormone replacement therapy in olds rats prevents sarcopenia by acting as a double-edged sword, antioxidant as well as myogenic. Our results highlighted that restoring a young redox status allowed to return to a normal functioning of the impaired pathways involved in sarcopenia. G6PDH appeared as a possible candidate by which growth hormone will restore the redox homeostasis.

It is important to note that the supplementation of GH that we have performed is a rather low one in that the aim is to return the levels to the normal physiological ones. If these results could be extrapolated to humans, one could suggest that the loosing of muscle mass observed in persons, even if they have performed exercise in their youth, could be prevented by hormone replacement therapy with low doses of GH. This interesting possibility remains to be studied in the clinical setting.

On the other hand, GH can have deleterious effects that should be studied. Although, we found positive effects on muscle, it would be interested to repeat a similar study and explore muscle function. In regards to the beneficial effect on mitochondriogenesis, it appears necessary to explore aerobic qualities.

In the second and third studies, we found that transgenic mice overexpressing G6PDH showed improved body composition that was characterized by a lower body weight, a decrease fat mass and an increase muscle mass. Moreover, we found better aerobic qualities and higher muscle strength in the G6PDHtg compared to the WT mice. In addition, we found a decreased DNA oxidative damage in the G6PDHtg compared to the WT mice. Although, surprisingly we did not find the expected protective effect against oxidative stress induced by exhaustive exercise and hyperoxia, an adverse effect was also not found.

Although the understanding mechanisms involved in these beneficial effects are still to be clarified, our results will provide new insight to develop strategies to fight against sarcopenia or in more large field to gain muscle mass, decrease fat mass and improved physical performance. However, before developing such strategies, more studies are needed to ensure the safety of an up-regulation of G6PDH. In this way, we are actually realizing a longevity curve. Since the G6PDH would support at the same time pro and antioxidant systems, we would explore if in response to repetitive exposure to pro-oxidizing conditions, the G6PDH overexpression does not lead to increase oxidative damage. In order to confirm the highlighted role of G6PDH in regulating muscle mass, doing a transgenic which would overexpress G6PDH only in skeletal muscle could be a better approach.

Finally, strategies targeting an up-regulation of G6PDH would not be undertaken in all populations particularly in those presenting a cancer risk since a possible oncogenic role of G6PDH has been highlighted.

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PUBLICATIONS AND PRIZES

Publications related to the thesis

- **Brioche T**, Kireev RA, Cuesta S, Gratas-Delamarche A, Tresguerres JA, Gomez-Cabrera MC, Viña J. **Growth hormone replacement therapy prevents sarcopenia by a dual mechanism: stimulation of protein synthesis and of antioxidant defenses.** J Gerontol A Biol Sci Med Sci. 2013 Dec 3. [Epub ahead of print]

- Mari Carmen Gomez-Cabrera, Beatriz Ferrando, **Thomas Brioche**, Fabian Sanchis-Gomar, and Jose Viña. **Exercise and antioxidant supplements in the elderly.** Journal of Sport and Health Science, Volume 2, Issue 2, June 2013, Pages 94-100

- **Brioche T**, Lemoine S, Gratas-Delamarche A, Gomez-Cabrera MC. *Invitation to publish a review in Free Radical Research intitled: Redox regulation of sarcopenia and exercise (in preparation)*

- The study 2 and 3 of this work are part of a bigger study that will be published in one article in collaboration with the team of Professor Viña J, Professor Seranna M and Professor Gratas-Delamarche

- **T Brioche**, MC Gomez-Cabrera, A Gratas-Delamarche, JA Tresguerres, and J Viña. **Growth Hormone replacement therapy in old rats protects against sarcopenia through antioxidant mechanisms.** Fundamental & Clinical Pharmacology. Volume 27, Issue Supplement s1, June 2013

Publications not related to the thesis

- Daniel Monleon[2]*, Rebeca Garcia-Valles*, Jose Manuel Morales[2], **Thomas Brioche[3]**, Gloria Olaso-Gonzalez, Raul Lopez-Grueso, Mari Carmen Gomez-Cabrera, Jose Viña. **Long term spontaneous exercise and plasma metabolome.** *Manuscript submitted tho the Journal of Apllied Physiology (Februrary 2014).*

- Helios Pareja-Galeano*, **Thomas Brioche***, Fabian Sanchis-Gomar, Angeles Montal, Carmen Jovaní, Cecilia Martínez-Costa, Mari Carmen Gomez-Cabrera, and Jose Viña. **Impact of exercise training on neuroplasticity-related growth factors in adolescents.** Accepted in Journal of Musculoskeletal and Neuronal Interactions, 2013

- Sanchis-Gomar F, Pareja-Galeano H, **Brioche T**, Martinez-Bello V, Lippi G. **Altitude exposure in sports: the Athlete Biological Passport standpoint**.Drug Test Anal. 2013 Sep 20. doi: 10.1002/dta.1539. [Epub ahead of print]

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- Fabian Sanchis-Gomar , Vladimir E. Martinez-Bello, Frédéric Derbré, Ernesto Garcia-Lopez, Rebeca Garcia-Valles, **Thomas Brioche**, Beatriz Ferrando, Sandra Ibanez-Sania, Helios Paleja-Galeano, Mari Carmen Gomez-Cabrera, José Viña. **Rapid hemodilution induced by desmopressin after erythropoietin administration in humans.** Journal of human sport and exercise. 6 (2) 2011

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- Fabian Sanchis-Gomar, Vladimir Martinez-Bello, Helios Pareja-Galeano, **Brioche T**, Mari Carmen Gómez-Cabrera. Chapter title: **An overview of doping in sports**. In Nutrition and Enhanced Sports Performance: Recommendations for Muscle Building. ELSEVIER

Prizes

- **PRIX DE MEILLEURE COMMUNICATION ORALE** du 8 ème congrès de Physiologie, de Pharmacologie et de Thérapeutique P2T, 2013, Angers (France)

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ANNEXE

Review

Exercise and antioxidant supplements in the elderly

Mari Carmen Gomez-Cabrera[a], Beatriz Ferrando[a], Thomas Brioche[b], Fabian Sanchis-Gomar[a], Jose Viña[a]

[a]Department of Physiology, Faculty of Medicine, University of Valencia, Fundacion, Investigacion Hospital Clinico Universitario/INCLIVA. Valencia, Spain.

[b]Laboratory "Movement Sport and Health Sciences", University of Rennes, Rennes, France.

Corresponding author: Mari Carmen Gomez-Cabrera
E-mail address: carmen.gomez@uv.es

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Abstract

Both exercise and aging increase Reactive Oxygen Species (ROS), which can result in damage to cells. Aging is the result of damage caused by ROS to the mitochondrial genome in post mitotic cells and numerous studies which have demonstrated an increase in ROS or their byproducts with exercise. ROS can cause oxidative stress as they overwhelm the antioxidant cellular defenses. Therefore interventions aimed at limiting or inhibiting ROS production, such as supplementation with antioxidant vitamins, should be able to reduce fatigue during muscle contraction and the rate of formation of aging changes with a consequent reduction of the aging rate and disease pathogenesis. However, it has been shown that ROS are essential signaling molecules which are required to promote the health benefits of exercise and longevity. In young individuals, ROS are required for normal force production in skeletal muscle, for the development of training-induced adaptations in endurance performance, as well as for the induction of the endogenous defense systems. Thus, taking antioxidants during training, in young athletes, seems to be detrimental. However, antioxidant supplementation may be expected to be beneficial and is receiving growing attention in the active old population. In this manuscript we review the literature associated with the main areas of interest in this topic.

Keywords: Oxidative stress, adaptations, aging, antioxidant enzymes, skeletal muscle, NF-κB, PGC-1α

1. Free radicals and exercise

Skeletal muscle generates Reactive Oxygen Species (ROS) during contractile activity. Research in this area started in 1954 when the emerging technology of electron spin resonance (ESR) spectroscopy was used to generate the first data showing that free radicals are present in muscle.[1] However, first suggestion that exercise was associated with an increase in lipid peroxidation by-products did not appear until the late 1970s.[2] The biological importance of this finding was unclear at the time. It was not until the early 1980s that researchers identified the first link between muscle function and free radical biology. ESR was again used to show that free radical content is elevated in isolated frog limb muscles stimulated to contract repetitively.[3] Shortly afterward, a ground-breaking report was published showing a 2- to 3-fold increase in free radical content of skeletal muscle from rats run to exhaustion[4]. These findings were associated with three aspects of damages that are now well-recognized: increased lipid peroxidation, decreased control of mitochondrial respiration, and decreased integrity of the sarcoplasmic reticulum. The same study showed that vitamin E deficiency inflated these three changes, indicating exercise-induced changes were sensitive to both free radical production and antioxidant buffering.[4] Since then, research in the area has grown rapidly. It is now clear that intense muscular contractile activity can result in oxidative stress not only in animals but also in humans. For instance, during the Tour de France, cyclists shown significant increases in plasma malondialdehyde (MDA) levels,[5] whereas similar results have been found in athletes after a marathon running.[6]

There are several potential tissue sources from which ROS may be produced during exercise: heart, lungs, white blood cells and skeletal muscle have been most studied.[7, 8] At the subcellular level, several sources of free radicals have been studied in skeletal muscle during exercise.[9] It has generally been assumed that an increase in oxygen consumption by mitochondria would lead to an increase in $O_2^{\bullet-}$ formation from complexe I and III. However, recent research suggests that mitochondria may not be the dominant source of ROS during exercise.[9,10] Rigorous exercise, especially eccentric contractions, may generate ROS via Nicotinamide Adenine Dinucleotide Phosphate, (NAD(P)H) oxidase from neutrophils and sarcolemma and secondarily, via myeloperoxidase. Interestingly, superoxide anions generated by these enzymes have been shown to regulate contractile function via calcium release in the cardiac muscle.[11] Phospholipase A2, an important enzyme involved in the metabolism of membrane polyunsaturated fatty acid during inflammation, has been identified as a modulator of cytosolic oxidant production in skeletal muscle.[12] Nethery and colleagues[13] showed that phospholipase A2 function is essential for the rise in intracellular ROS that occurs during repetitive, fatiguing contractions. Cyclooxygenase and lipoxygenase are involved in ROS production with

phospholipase A2. The role of xanthine oxidase (XO) in oxidant generation during high-intensity intermittent exercise has long been recognized.[6,14,15] Depletion of ATP during demanding muscle contraction results in an accumulation of hypoxanthine and xanthine and conversion of xanthine dehydrogenase to XO. These conditions set the stage for generating $O_2^{\bullet-}$ when oxygen is replenished to relatively hypoxic muscle.[16] Administration of allopurinol or oxypurinol, a drug widely used in the clinical practice to treat gout due to its inhibitory effect on XO, has been shown to decrease muscle oxidative stress after exhaustive exercise both in humans and in rats.[5,17,18] Finally, nitric oxide (NO) is generated continuously within skeletal muscle by NO synthase (NOS) with an important function to regulate vascular smooth muscle tone.[19] Heavy muscle contraction can increase NO production via activation of eNOS or iNOS which may have some detrimental effect due to the danger of forming highly reactive peroxynitrite.[20] A schematic diagram of the sources of free radicals in skeletal muscle is in Fig. 1.

2. The role of antioxidants in the modulation of skeletal muscle adaptations to exercise

As mentioned in the previous section free radical production during muscle contraction has been related to several aspects of damage. Thus, the idea of the deleterious effects of free radicals has been firmly entrenched in the minds of scientists during the last 30 years[10]. It has been generally accepted that increasing the intracellular levels of antioxidants within a muscle cell should provide protection against these oxidizing agents and reduce fatigue.[21-23] During the early 1980s, several research groups investigated the role of antioxidant nutrients in the protection of cells and organelles from radical-mediated oxidative damage.[24] In 1983 Jackson and colleagues[25] examined the role of ROS as damaging agents to muscle and the possible beneficial effects of vitamin E in reducing exercise-induced damage. These studies stimulated the interest of many laboratories to investigate whether antioxidant nutrients could retard both tissue damage and muscle contractile dysfunction that occurred during some forms of muscular exercise. There is no doubt that antioxidant supplementation decreases the markers of oxidation in tissues.[26-28] Based on these data many athletes consume quantities of vitamins E and C well above the recommended dietary allowances.[29] Vitamin C is one of the biggest-selling nutrients in the U.S. vitamin and mineral market, with predominantly healthy people (including athletes) topping the buyers' list.[30] However, the positive effects of dietary antioxidants against contraction-induced muscle damage and muscular fatigue are not commonly observed.[7] Although the generation of ROS is an inevitable event associated with muscle contraction during physical exercise, we now know that its production is determined

by the intensity, frequency, and duration of the exercise protocols. It has been shown that exercise training reduces the oxidative stress of exercise, trained athletes show less evidence of lipid peroxidation for a given bout of exercise and an enhanced defense system in relation to untrained subjects.[10] Thus exercise training can be considered as an antioxidant.[10] The dramatic ability of the body to increase antioxidant capacity with acute and chronic exercise has been described in several tissues.[31-33] There is now an appreciation that the ROS generated during muscle contraction have a physiological role in the adaptations to exercise. In response to the free radical assault, the cell has developed a number of antioxidant defense systems. There is growing evidence that the continued presence of a small stimulus such as low concentrations of ROS is in fact able to induce the expression of antioxidant enzymes and other defense mechanisms. The basis for this phenomenon may be encompassed by the concept of hormesis,[34] which can be characterized as a particular dose-response relationship in which a low dose of a substance is stimulatory and a high dose is inhibitory. In this context radicals may be seen as beneficial as they act as signals to enhance defenses rather than deleterious as they are when cells are exposed to high levels of these radicals. Recently the hormesis theory has been extended to the ROS generating effects of exercise.[35,36] In skeletal muscle hydrogen peroxide at a low concentration increases calcium release from the sarcoplasmic reticulum and force production, whereas a massive increase in hydrogen peroxide concentration results in a sharp decrease in force output.[37] Animals frequently exposed to exercise (chronic training) have shown less oxidative damage after exhaustive exercise than untrained ones. This is largely due to the up-regulation of endogenous antioxidant enzymes such as mitochondrial superoxide dismutase, glutathione peroxidase, and γ-glutamylcysteine synthetase.[38] We have shown that this up-regulation is mediated by redox sensitive transcription factors such as Nuclear Factor κB (NF-κB).[6,17,39] Thus, the convenience of supplementing antioxidant vitamins in the sport population is nowadays an object of debate. In fact training studies conducted to determine whether antioxidant vitamins improve exercise performance have generally shown that supplementation is useless[40-44] or even negative.[45] Several studies suggest that antioxidants may have detrimental effects on performance.[46-49] We have found that vitamin C supplementation decreases training efficiency because it prevents exercise-induced mitochondrial biogenesis.[50] These results have been confirmed by other research groups.[51, 52] A large proportion of athletes, including elite athletes, take vitamin supplements, often large doses, seeking their beneficial effects on performance.[53] The complete lack of any positive effect of antioxidant supplementation on

physiologic and biochemical outcomes consistently found in human and animal studies raises questions about the validity of using oral antioxidant supplementation in the sport population.[45]

3. Free radicals and exercise at old age

There are many theories of ageing.[54] One of the most prominent theories to explain ageing is the free radical theory of ageing which was initially proposed by Harman[55] in the 1950s. It proposes that free radicals derived from oxygen are responsible for damage associated with ageing. The antioxidant systems are unable to counterbalance all the free radicals continuously generated during the life of the cell. This results in oxidative damage in the cell and thus in tissues. There is a great deal of experimental proof in support of this theory. The findings in the laboratory of Britton Chance that ~2% of oxygen consumed by mitochondria in state 4 is converted to hydrogen peroxide underlined the role of mitochondria in ROS production.[56] These experiments led to Jaime Miquel to refine the free radical theory of aging and in the 1970s he formulated the mitochondrial free radical theory of aging. The main contributions of Miquel were: emphasized the importance of mitochondrial DNA as a target of oxidants produced during aging, and pointing out that mitochondrial biogenesis might be impaired in aging.[57]

The mitochondrial theory of aging, although recently questioned,[58] has been tested in various laboratories and there are many published papers in support of this theory.[59,60] The continuous free radical generation by mitochondria during the whole life span, causes a chronic oxidative stress that plays a critical role in aging. Thus, aging is associated with free radical generation in several tissues including skeletal muscle.[55,61] Senile sarcopenia is defined as the loss of muscle mass and force associated to aging.[62] It has been estimated that muscle fibre loss occurs as early as at age 25 and that at age 80 total muscle fibre number shows a decrease of almost 40%.[63] ROS have been proposed to be involved in the underlying mechanism of age-induced sarcopenia. As a response to this oxidative stress skeletal muscle antioxidant enzyme activities are increased with old age.[64] However, protein and mRNA levels of these enzymes are found to be either decreased or unaltered in the aged muscle.[65] Alterations in the NF-κB cell signaling pathway seem to be responsible for this impairment. NF-κB is believed to be constitutively activated in skeletal muscle at old age. This increased transcription seems to be part of a general cellular adaptive response aimed at providing protection against subsequent,

damaging insults.[66] However, chronic activation of NF-κB leads to the higher basal expression of pro-inflammatory cytokines, chemokines, and adhesion molecules. In fact, it has been identified as a main etiological reason for aged-related muscle wasting and sarcopenia.[67] However, there is a failure to fully activate NF-κB in the skeletal muscle of old animals following contractile activity[66] (Fig. 2). The mechanisms responsible for this fall are unclear. Thus, during aging there is an impairment in the signal transduction of antioxidant gene expression in response to oxidative stress.[68]

Other relevant co-activator affected by aging is peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α). It acts as a master regulator of energy metabolism and mitochondrial biogenesis by coordinating the activity of multiple transcription factors.[69] Aging has been associated, in skeletal muscle, with reductions in mitochondrial oxidative phosphorylation activity, mitochondrial DNA mutations, reductions in mitochondrial DNA content, decreased activities of the mitochondrial electron transport chain, and altered apoptotic signalling.[70] Thus, the promotion of mitochondriogenesis is critical to prevent aging in skeletal muscle. We have recently shown that muscle from old rats present a marked loss in mitochondriogenesis and that this may be due to a lack of induction of PGC-1α.[71] We found a striking similarity between the response to exercise training in *PGC-1α* knock-out (KO)mice and in old rats. In young rats, PGC-1α was activated in skeletal muscle not only by training but also by cold exposure or triiodothyronine. However, in the old animals we found an age-associated lack of expression of PGC-1α in response to exercise training or to any of the other stimuli tested in rat skeletal muscle. Our study highlighted the importance of maintaining a normal PGC-1α responsiveness to maintain normal muscle function (Fig. 2).

4. The role of antioxidants in the modulation of the ageing process

An important characteristic of the free radical theory of aging is that, it opens up room for intervention, because if radicals are causing oxidative damage to cells and this is associated with age-associated damage, then administration of antioxidants could delay ageing and perhaps even prevent age-associated diseases. Cutler[72] observed that several antioxidants such as vitamin E, uric acid, cellular plasmin, or superoxide dismutase in several organisms show an inverse relationship with the basal metabolic rate and with the maximal longevity of the species. Thus, he proposed that the maximal life span should be correlated with the antioxidant capacity of cells. In keeping with this line of thought Orr and Sohal[73] observed that double transgenic Drosophila over-expressing Cu/Zn-superoxide dismutase and catalase

show less oxidative stress and longer life span, both mean and maximal. Moreover, they found that the process of ageing was slowed. Indeed, the transgenic Drosophila showed a lower loss in physical activity and less markers of damage in proteins. However, the assumption that antioxidant supplements are in general good for one´s health has been proof to be wrong. A critically important point is the relationship between the various antioxidants in cells. Persons with defects in absorption of vitamin E or with low glutathione levels show different conditions but not an accelerated ageing. In fact, using high doses of vitamin E in age-related diseases such as Alzheimer's, has been questioned after the publication of some studies which show that its administration is detrimental for the patients.[74] The evidence on the detrimental effects of antioxidant supplementation when given to patients and healthy people is robust. In 2007, Bjelakovic et al.[75] looked at data from sixty-seven studies on antioxidant supplements and they concluded that beta carotene, vitamin A, and vitamin E supplementation seemed to increase the risk of death. This data confirmed previous reports showing that long-term vitamin E supplementation may increase the risk for heart failure in patients with vascular disease or diabetes mellitus.[76]

5. Exercise and antioxidant supplementation at old age

The beneficial effect of physical activity for the promotion of health and curing of diseases among individuals of all ages is beyond all doubt. Strong scientific evidences link physical activity to several benefits, including the promotion of health span and not only of lifespan. Although physical activity has many well-established health benefits,[77] aging and strenuous exercise are associated with increased free radical generation in the skeletal muscle.[78] Thus, whether exercise would worsen the skeletal muscle oxidative stress in aged population has been an object of debate. Research evidence indicates that senescent organisms are more susceptible to oxidative stress during exercise because of the age-related ultrastructural and biochemical changes that facilitate ROS generation.[78] Aging also increases the incidence of muscle injury, and the inflammatory response can subject senescent muscle to further oxidative stress. Furthermore, muscle repair and regeneration capacity is reduced at old age that could potentially enhance the cellular oxidative damage.[78] Thus, several researchers consider that dietary antioxidant supplementation should be beneficial in the old physically active population.[79] Recent studies suggested a beneficial relationship between antioxidant vitamin (e.g., vitamin C) intake and physical performance in elderly people.[80] It has been shown that intake of resveratrol, together with habitual exercise, is beneficial for suppressing

the aging-related decline in physical performance.[81] Moreover it has been shown that antioxidant supplementation improves indices of oxidative stress associated with repetitive loading exercise and aging and improves the positive work output of muscles in aged rodents.[82] Bobeuf and co-workers[83] found that 6 months of resistance training combined with antioxidant supplementation significantly increased fat-free mass in older adults. However, these results have not been confirmed by other studies. Nalbant and collaborators[84] found that 6 months of vitamin E supplementation had no additive effect beyond that of aerobic training on indices of physical performance and body composition in older sedentary adults. Regarding bone density it has been shown that combination of resistance training with antioxidant vitamins supplementation does not seem to produce synergistic effects on the prevention of osteoporosis.[85] The convenience of supplementing with antioxidant vitamins in the old sport population is nowadays, as in the young population, an object of debate. Richardson's research group identified a clinically significant paradoxical cardiovascular response to exercise training and antioxidant supplementation in the elderly.[86] Antioxidant administration, after exercise training, blunted training-induced reduction in blood pressure as well as the exercise-induced improvements in flow-mediated vasodilation. The paradoxical effects of these interventions suggest a need for caution when exercise and acute antioxidant supplementation are combined in elderly mildly hypertensive individuals. Thus, the paradoxical effects of antioxidant supplementation, when combined with exercise training, reveal an intriguing, but complex, relationship between aging, exercise, and oxidative stress. More research for a better clarification of the field is required.

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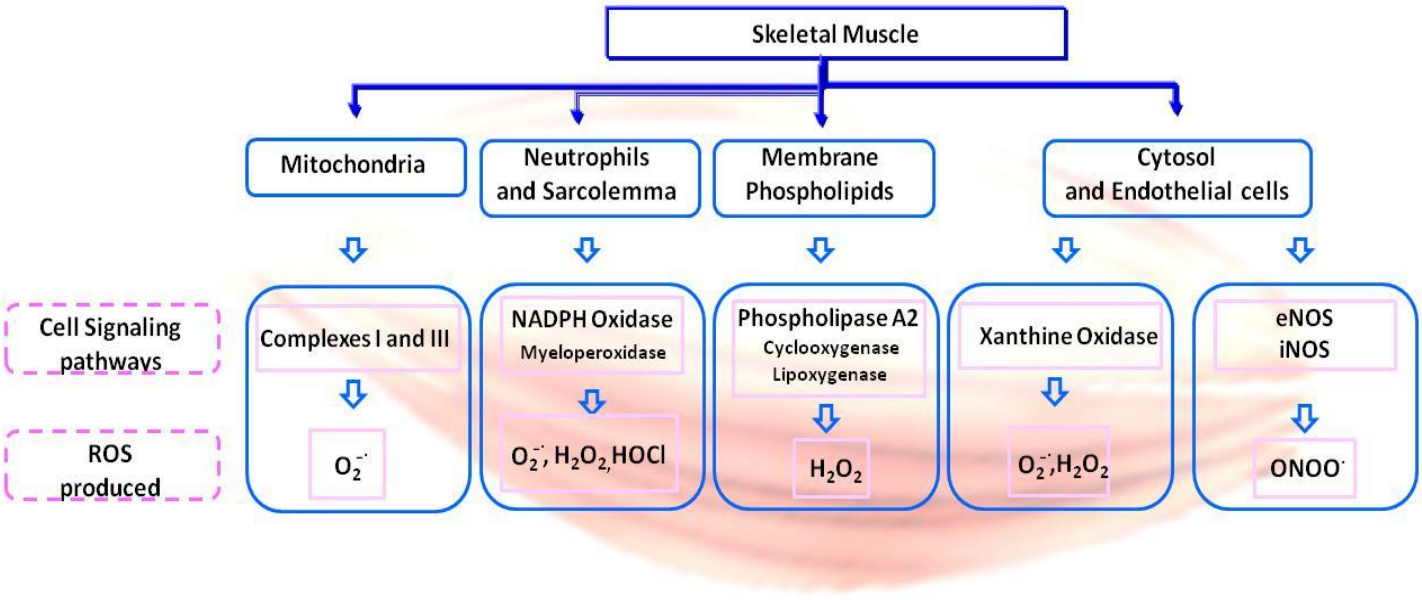

Fig. 1. Potential sites for the production of free radicals in skeletal muscle. There are several sources of free radicals in skeletal muscle. They are located in mitochondria, cytosol, sarcolemma, and endothelial cells. ROS = Reactive Oxygen Species NOS = Nitric Oxide Synthase

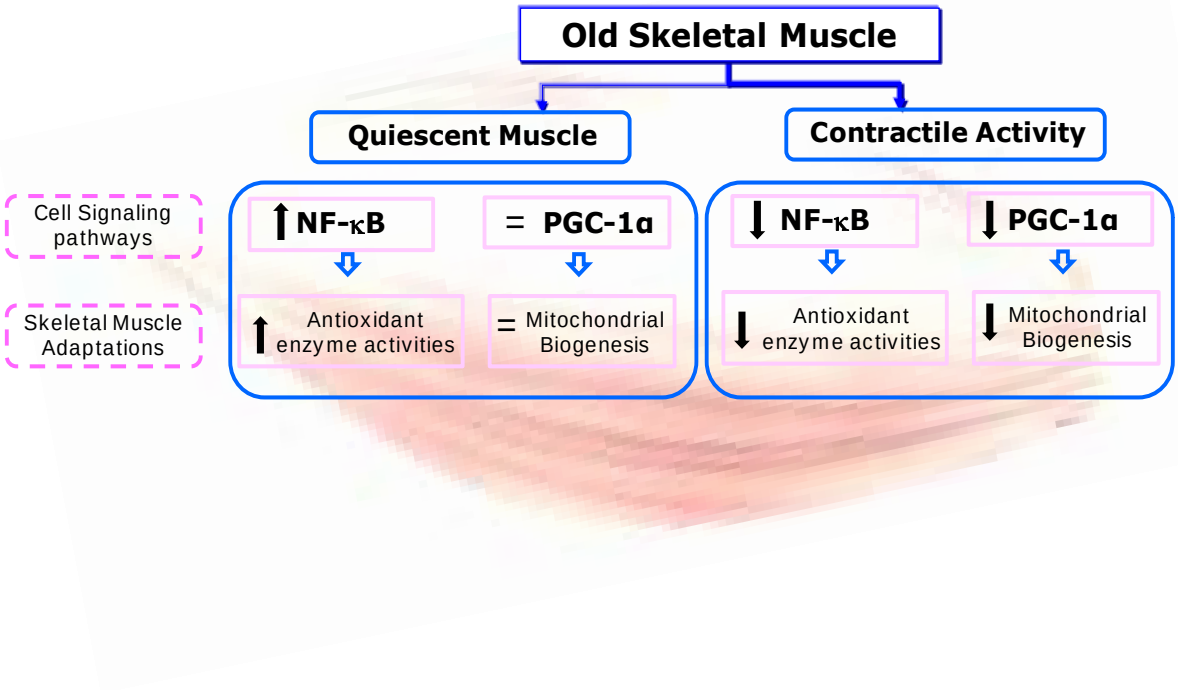

Fig. 2. Redox sensitive cell signaling pathways altered in skeletal muscle at old age. Nuclear Factor κB (NF-κB) is constitutively activated in skeletal muscle at old age which leads to an impairment in the oxidative stress response. However, there is a failure to fully activate NF-κB in the skeletal muscle of old animals following contractile activity. The attenuated mitochondrial biogenesis reported in both the quiescent and stimulated skeletal muscles at old age compared to young is at least partially due to an attenuation of Peroxisome Proliferator-Activated Receptor-γ Coactivator 1α (PGC-1α) signaling ability.

Abstract	Résumé
Aging is characterized by a decrease in muscle mass and strength causing a deterioration of physical performance, called sarcopenia. Muscle atrophy can be explained by a negative protein turnover, impaired mitochondrial dynamics, a decreased muscle regeneration capacity and myonuclei apoptosis. A decreased production of anabolic hormones and a chronic oxidative stress (OS) which leads to excessive oxidative damage, would be involved in these alterations. Physical exercise and hormone replacement therapies are effective to combat sarcopenia. The restoration of a redox homeostasis may play a central role in their beneficial effects and would involve an up-regulation of the glucose-6-phosphate dehydrogenase enzyme. The main objectives of this thesis were to determine *in vivo* to what extent a pro-oxidant redox status in aged muscle may modulate signaling pathways involved in sarcopenia, and to investigate whether return to their normal functioning requires a restoration of the redox homeostasis. The third objective was to identify actors and their possible cellular mechanisms in the maintenance and/or the restoration of the redox status. In a first study in old rats, we first confirmed that sarcopenia is associated with OS. In a second time, we found that a growth hormone replacement therapy in old rats prevents sarcopenia by acting as a double-edged sword, antioxidant as well as myogenic, associated with an up-regulation of G6DPH. In a second study, we found that transgenic mice overexpressing G6PDH showed improved body composition and physical performances. In a third study, we found that overexpression of G6DPH improves DNA oxidative damage in resting conditions. However, the expected protective effect of G6PDH overexpression against oxidative stress induced by pro-oxidizing stimuli was not present.	Le vieillissement est caractérisé par une diminution de la masse et la force musculaire entraînant une détérioration des performances physiques, appelée sarcopénie. L'atrophie musculaire peut être expliquée par un turnover protéique négatif, une détérioration des dynamiques mitochondriales, une diminution de la capacité de régénération du muscle ainsi que par l'apoptose des noyaux musculaires. La diminution de la sécrétion d'hormones anabolisantes et un stress oxydant (OS) chronique conduisant à des dommages oxydatifs excessifs, seraient impliqués dans ces modifications. L'exercice physique et les thérapies de remplacement hormonales sont efficaces pour lutter contre la sarcopénie. Une restauration de l'homéostasie redox pourrait avoir un rôle central dans la lutte contre la sarcopénie et impliquerait une activation de la glucose-6-phosphate déshydrogénase. Les principaux objectifs de cette thèse étaient de déterminer *in vivo,* si un SO chronique dans le muscle âgé altère les voies de signalisation impliquées dans la sarcopénie, et de chercher si le retour à un fonctionnement normal de ces voies nécessite une restauration de l'homéostasie redox. Certains paramètres et leurs mécanismes pouvant intervenir sur le maintien ou la restauration du SO ont été recherchés. Dans une première, nous avons confirmé que la sarcopénie est associée au OS chez le rat. Puis nous avons constaté qu'un traitement à l'hormone de croissance chez le rat peut prévenir la sarcopénie via un effet antioxydant et myogénique, associé à une activation de la G6DPH. Une seconde étude a monté que des souris transgéniques surexprimant la G6PDH présentaient une amélioration de la composition corporelle et des performances physiques. Une dernière étude a montré que la surexpression de G6DPH diminuait les dommages oxydatifs de l'ADN au repos. De façon surprenante, la surexpression de la G6PDH n'a pas d'effet protecteur vis à vis du SO induit par les divers stimuli pro-oxydants.

Keywords: sarcopenia, oxidative stress, exercise, growth hormone, skeletal muscle, G6DPH	**Mots-clés:** sarcopénie, stress oxydant, exercice, hormone de croissance, muscle, G6DPH

Laboratoire « Mouvement, Sport, Santé »

Avenue Robert Schuman 35170 Bruz

Tél : + 33(2) 90 09 15 80

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Thomas Brioche

Préparée au sein des laboratoires Free Radical and Antioxidant Research Group (Université de Valencia, Espagne) et Mouvement, Sport, Santé (Université Rennes 2 – ENS Rennes, France)

Sarcopenia: Mechanisms and Prevention
Role of Exercise and Growth Hormone

Involvement of oxidative stress and Glucose-6-phosphate dehydrogenase

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devant le jury composé de :

Angèle CHOPARD
Professeur - UMR 866 DMEM, Montpellier / rapporteur

Damien FREYSSENET
Professeur - Laboratoire de Physiologie de l'Exercice, Saint-Etienne / rapporteur

Isabelle PETROPOULOS
Professeur - Laboratoire de Biologie Cellulaire du Vieillissement, Paris / Examinateur

José VINA
Professeur - Laboratoire FRAG, directeur de thèse

Arlette GRATAS-DELAMARCHE
Professeur - Laboratoire M2S, directrice de thèse

Mari-Carmen GOMEZ-CABRERA
Professeur - Laboratoire FRAG, Co-directrice de thèse

Sophie LEMOINE-MOREL
Maître de conférences - Laboratoire M2S, Co-directrice de thèse

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RESUMEN EN CASTELLANO

I) Introducción

Hay aproximadamente 80 años, MacDonald Critchley fue el primero en reconocer que la masa muscular disminuye durante el envejecimiento y se dio cuenta de que esta pérdida era aún más importante en los músculos de las extremidades superiores y inferiores (Critchley 1931). Casi sesenta años después, en 1988, durante una conferencia en Albuquerque (Estados Unidos) centrado en la evaluación del estado de salud de los ancianos, Rosenberg señaló que "ninguna disminución con la edad es más dramática o potencialmente más funcionalmente importante que la disminución de la masa muscular". Hizo hincapié en que para reconocer este fenómeno por la comunidad científica, se necesitaba un nombre y propuso el término "sarcopenia" (del griego "sarx": carne + "penia": perdida). A partir de entonces, la sarcopenia se definió como la pérdida general y progresiva de la masa muscular que se produce con la edad (Roubenoff & Hughes 2000). Sin embargo, esta definición no fue aceptada por todos los clínicos y los investigadores y ha evolucionado hasta hace unos años. Por último, el consenso actual define la sarcopenia como un "síndrome geriátrico caracterizado principalmente por una disminución de la masa muscular associada a una disminución de la fuerza muscular y del rendimiento físico" (Muscaritoli et al. 2010, Cruz-Jentoft et al. 2010 Fielding et al., 2011; Morley et al. 2011).

Gracias a los progresos sociales, tecnológicos y médicos, la esperanza de vida no dejó de aumentar desde el siglo 19 en nuestras sociedades occidentales modernas, conduciendo a un envejecimiento general de la población. Actualmente, mundialmente, está previsto que el número de personas mayores habrá doblado en 2050, pasando del 11 % de la población al 22 % (la ONU 2007). Inevitablemente, debido a este envejecimiento de la población, la prevalencia de la sarcopénie está creciendo, y actualmente consideramos que entre un cuarto y la mitad de los hombres y las mujeres de 65 años y más son susceptibles de ser sarcopenicos (Janssen 2004). Las consecuencias del crecimiento de la prevalencia de la sarcopenia están generalmente consideradas como catastróficas sobre los costes de Sanidad Pública.

Así, el coste total de la sarcopenia al sistema americano de salud ha sido estimado a cerca de 18,4 mil millones de dólares (Janssen y al. 2004). De ahora en adelante, por el hecho de que las personas de edad de más de 69 años representan la rebanada de la población americana que crece más rápidamente, parecería que de ahora en adelante este coste sólo aumente (Manton y Vaupel, 1995). Estos gastos de salud son asociados a un deterioro general del estado físico que conduce a un riesgo aumentado de caídas, una incapacidad

progresiva en cumplir actividades elementales de la vida cotidiana y la pérdida de autonomía de los ancianos (Goodpaster y al. 2006; Delmonico y al. 2007).

Sin embargo, varias estrategias son reconocidas como eficacias para prevenir, retrasar o tratar la sarcopenia. Así, desarrollar terapias ayudaría no solo a mejorar la calidad de vida de las personas sarcopenicas, sino que también reduciría los costes económicos asociados con la sarcopenia, lo que sería benéfico a la sociedad entera. Actualmente, el ejercicio físico es innegablemente la estrategia más eficaz en la lucha contra la sarcopenia, porque puede conducir a aumentar la masa muscular, la fuerza y el rendimiento físico (Pillard et al. 2011; Di Luigi et al. 2012; Wang & Bai 2012; Montero & Serra 2013). Sin embargo, la puesta en ejecución de escala grande de tal intervención es trabada por la falta de motivación de la inmensa mayoría de las personas. Además, muchas personas mayores no pueden caminar o tienen comorbilidades como la osteoartritis moderada a grave (Bennell y Hinman 2011) o ciertas formas de enfermedad cardiovascular inestable que les exclue de la participación en protocolos de ejercicio (Williams et al. 2007). Para superar estos obstáculos, el desarrollo de terapias alternativas como las estrategias antioxidantes y las terapias de reemplazo hormonales (testosterona y hormona del crecimiento) parece necesario.

El músculo esquelético es un órgano que tiene propiedades específicas que le dan un papel central en la locomoción, la realización de actividades de la vida diaria, el mantenimiento de la postura y el equilibrio. Para garantizar estas funciones esenciales, el músculo debe tener una masa suficiente que todo el mundo debe tratar de conservar. Como se ha descrito anteriormente, algunas de las más graves consecuencias del envejecimiento son sus efectos en el músculo esquelético, en particular, la pérdida progresiva de la masa y la función que tienen un impacto en la calidad de vida y, en última instancia, en la esperanza vida (Cruz-Jentoft 2012).

Actualmente los mecanismos subyacentes a la sarcopenia no están aún bien definidos, y por lo tanto, siguen siendo objeto de muchas investigaciónes. Sin embargo, un turnover proteico negativo (Combaret et al. 2009), una alteracion de las dinámicas mitocondriales (Calvani et al. 2013), una disminución de la capacidad de regeneración muscular (Snijders et al. 2009; Hikida 2011), así como la exacerbación de la apoptosis de los núcleos musculares (Marzetti et al. 2012) se consideran generalmente como los mecanismos celulares implicados en la atrofia muscular que conduce a la sarcopenia.

Estos mecanismos son ellos mismos dependientes de una multitud de factores celulares y sistémicos, tales como la disminución de la producción de hormonas anabolisantes (GH, IGF-1, la testosterona, la insulina). Los vínculos y las interacciones entre estas

secreciones hormonales disminuidas y las disfoncciones celulares mencionadas prevviamente siguen siendo en gran parte desconocidos. Un posible candidato podría ser el estrés oxidativo crónico relacionado con la edad (Semba et al. 2007; Safdar et al. 2010).

Por lo tanto, el músculo sarcopenico presenta una sobreproducción de especies no radicales o radicales derivadas del oxígeno y del nitrógeno (RONS) (Capel et al. 2004; Capel, Rimbert, et al. 2005; Capel, Demaison, et al. 2005; Chabi et al. 2008; Jackson et al. 2011; Andersson et al. 2011; Miller et al. 2012). Esta sobreproducción de RONS es principalmente debida a disfunciones mitocondriales (Capel, Rimbert, et al. 2005; Chabi et al. 2008), y un aumento de la actividad de la xantina oxidasa (Lambertucci et al. 2007; Ryan et al. 2011), y conduce a un aumento de los daños oxidativos a los diversos componentes celulares y moleculares de la del músculo esquelético. Estos daño oxidativos reflejan la incapacidad de los sistemas antioxidantes a soportar la sobreproducción de RONS y atestiguan del desequilibrios de la balanza "pro-oxidante/antioxidante" conduciendo a una alteracion de la homeostasis redox (Jones 2006). Pareceria que la restauración de la homeostasis redox por algunas de las estrategias contra la sarcopenia implica un aumento del contenido proteico y/o de la actividad de la glucosa-6-fosfato deshidrogenasa (G6PDH) en el músculo (Kovacheva et al. 2010; Sinha-Hikim et al. 2013). La G6PDH es la enzima limitante de la vía del los pentosas fosfatos conocida para ser la fuente de NADPH de ciertos sistemas antioxidantes (Scott y al. 1993). Además, algunos datos *in vitro* y *in vivo* sugieren que la G6DPH jugaría un papel importante dentro de la regulación de la masa muscular. Sin embargo, estos datos necesitan confirmarcion.

Objetivos:

En este contexto, la presente tesis intentará responder a tres objetivos generales. El primer objetivo es determinar *in vivo* cómo un estado redox pro-oxidante debido al envejecimiento en el tejido muscular puede modular las vías de señalización implicadas en los mecanismos moleculares que subyacen a la sarcopenia. El segundo objetivo es mostrar que un retorno al funcionamiento normal de estas vías de señalización se asocia con una restauración de la homeostasis redox. Por último, el tercer objetivo de esta tesis es identificar actores y posibles mecanismos por los cuales se podría mantener la homeostasis redox.

Los objetivos especificos son los sigientes:

- Determinar si el estado prooxidante crónico en el músculo esquelético de ratas envejecidas puede modular las vías de señalización conduciendo a la sarcopenia, implicadas en la síntesis de proteínas y la proteolisis, sino también en la regeneración muscular y la mitochondriogenesis. Nuestra hipótesis es que el estrés oxidativo conduciría a una down-regulación de las vías de señalización de PI3K/Akt/mTOR y PGC-1α/Tfam/Nrf-1, y una up-regulación de los marcadores del sistema ubiquitina-proteasoma, así como de los inhibidores de la regeneración muscular (Estudio 1).

- Determinar si y por cuales mecanismos un tratamiento substitutivo a la hormona de crecimiento permite prevenir la sarcopénia en ratas envejecidas. Hacemos dos hipótesis.

    1) La GH a través de un aumento de las concentraciones de IGF-1 circulante permitiría la restauración de un funcionamiento normal de la vía de señalización PI3K/Akt/ mTOR mientras disminuiría la expresión de varios actores del sistema ubiquitine-proteasome dependiente y de inhibidores de la regeneración muscular. Un efecto posible sobre el mitochondriogenèse es también esperado (estudio 1).

    2) Estos efectos benéficos serían asociados con un mejoramiento del estado redox en particular gracias a la up-regulación de ciertas enzimas antioxidantes (estudio 1).

- Determinar *in vivo* en un modelo de ratón sobreexpresando la glucosa-6-fosfato deshidrogenasa, el papel de esta enzima en la regulación de la composición corporal (masa muscular y masa grasa) y su impacto en el rendimiento físico (fuerza, consumo máximo de oxígeno y resistencia) (Estudio 2).

- Determinar si la sobreexpresión *in vivo* de G6PDH mejora el estado redox en reposo y protege en situaciones pro-oxidantes (ejercicio exhaustiva y hiperoxia) (Estudio 3).

II) Contribución Personal

<u>Estudio 1:</u> La terapia de reemplazo con hormona de crecimiento previene a la sarcopenia mediante un doble mecanismo: mejora el recambio de proteínas y las defensas antioxidantes.

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Growth hormone replacement therapy prevents sarcopenia by a dual mechanism: improvement of protein balance and of antioxidant defenses

Authors: Brioche T[1,2], Kireev RA[3], Cuesta S[3],Gratas-Delamarche A[2], Tresguerres JA[3], Gomez-Cabrera MC[1], and Viña J[1]

Affiliations:[1]Department of Physiology. University of Valencia. Fundacion Investigacion Hospital Clinico Universitario/INCLIVA. Spain.

[2]Laboratory "Movement Sport and health Sciences", University Rennes. France

[3]Department of Physiology. University Complutense of Madrid. Spain

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Address correspondence to:

Jose Viña, Professor, MD (Corresponding author)

Department of Physiology. Faculty of Medicine. University of Valencia.

Av. Blasco Ibañez, 15, Valencia

46010 Spain

Phone: (34) 96 386 46 50

Fax: (34) 96 386 46 42

Email: jose.vina@uv.es

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INTRODUCCIÓN

El consenso actual define la sarcopenia como un síndrome geriátrico primero caracterizado por una disminución de la masa muscular, que agravándose participará en una disminución de la fuerza muscular y el deterioro del rendimiento físicofísico" (Muscaritoli et al. 2010; Cruz-Jentoft et al. 2010; Fielding et. al 2011; Morley et al. 2011) dando lugar a la aparición de numerosas comorbilidades tales como una discapacidad física, un deterioro de la calidad de vida y la reducción de la esperanza de vida (Evans 1995). Esta pérdida de masa muscular se produce a un ritmo de 3 a 8% por década después de la edad de treinta años, y este porcentaje aumenta después de sesenta años (Holloszy 2000). Estimaciones recientes indican que entre un cuarto y la mitad de los hombres y mujeres mayores de sesenta y cinco años están considerados sarcopénico (Janssen 2004). La sarcopenia juega un papel importante en el desarrollo de la fragilidad, y aumenta gradualmente el riesgo de caídas y reduce la capacidad de las personas para llevar a cabo actividades de la vida diaria (Evans 1995). Por último, los sujetos con sarcopenia en los estados más avanzados pierden su independencia y terminan siendo institucionalizado (Wolfe 2006)

Ciertas hormonas son conocidas para tener un efecto sobre la masa, la fuerza y la función muscular (Cruz-Jentoft 2012). Entre ellas, la hormona de crecimiento (GH) es una de las más estudiadas (Cruz-Jentoft 2012). Los niveles de GH son habitualmente reducidos en los sujetos de edad así como la amplitud y la frecuencia de su secreción pulsatile (Cruz-Jentoft 2012). Así, ha sido emitida la hipótesis que la GH sería eficaz en la prevención de la pérdida de la masa muscular durante el envejecimiento (Giannoulis y al. 2012).

En nuestro estudio, procuramos elucidar el papel de una terapia de sustitución con GH en ratas viejas comparando animales viejos (24 meses) con animales jóvenes (3 meses) y con animales viejos tratados con GH (8 semanas, 2 mg/kg repartidos en 2 inyecciones subcutáneas diarias). Nos centramos sobre cuatro de los principales mecanismos implicados en la aparición y la progresión de la sarcopenia: la alteración de la biogénesis mitocondrial, el aumento del estrés oxidativo, el aumento de la degradación de las proteínas y la disminución de su síntesis (Doherty 2003; Derbré y al. 2012).

En este estudio, presentamos pruebas de que la restauración del perfil de GH es una buena intervención para mejorar o mantener la masa muscular esquelética en los animales más viejos.

RESULTADOS/DISCUSIÓN

Efecto del envejecimiento y de la terapia de sustitución de GH sobre la composición corporal en ratas

A pesar del gran número de estudios para evaluar los efectos de la suplementación con GH sobre la masa muscular, resultados controvertidos en la literatura mantienen el debate sobre si la GH puede o no puede ser utilizada para luchar contra la sarcopenia (von Haehling et al. 2012). Los resultados controvertidos pueden ser explicados por las diferencias metodológicas tales como las dosis utilizadas. Dosis elevadas de GH son frecuentemente la causa de efectos adversos (Papadakis y al. 1996; Holloway y al. 1994). Es por eso que en nuestro estudio, utilizamos dosis relativamente débiles. Nuestros resultados mostraron que las concentraciones plasmáticas de IGF-1 eran más débiles en los animales viejos que en los animales jóvenes pero el tratamiento con GH permitió restablecer valores comparables a los animales jóvenes. Sin embargo, los animales mayores tratados con GH mostraron un aumento de peso, significativamente diferente de la pérdida de peso que occurio en ratas mayores no tratadas. Esta pérdida se debe principalmente a los cambios en la masa corporal magra porque el índice de gravedad específica (SGI: índice calculado a partir de una técnica de pesaje hidrostática) paso de 5 en los animales jóvenes a 3 en los animales mayores. El SGI es un indicie que evalua la masa magra y la masa adiposa; cuanto más es elevado, más la masa magra del animal es elevada. Nuestros datos también muestran que la administración de GH aumenta considerablemente el SGI en las ratas viejas, lo que significa que la GH, a través de sus propiedades anabolizantes, anti-adipogenicas y lipoliticas, es capaz de aumentar la masa muscular y reducir la masa adiposa (Castillo y al. 2004; Castillo y al. 2005).

También se evaluó la atrofia de los músculos gastrocnemius pesandolos, y se encontró una reducción del 30% del peso de los músculos de los animales viejos en comparación con los animales jóvenes. Esta atrofia fue completamente prevenida en las ratas viejas tratadas con GH.

El efecto antioxidante de la terapia de sustitución de GH

La teoría del envejecimiento de los radicales libres proporcionó una base teórica para el diseño de experimentos para entender el envejecimiento. (Gomez-Cabrera et al. 2012). Está bien establecido que la up-regulacion de las defensas antioxidantes endógenas es un mecanismo eficaz para prevenir el daño oxidativo asociado con la producción excesiva de

radicales libres (Gomez-Cabrera, Domenech & Viña 2008; Gomez-Cabrera, Domenech, Romagnoli, et al. 2008). Los efectos de la GH sobre la sarcopenia se han estudiado ampliamente (Brill et al. 2002; Papadakis et al. 1996), pero hasta ahora, jamás han sido centrados sobre la prevención de los daños de los radicales libres. Una de las principales conclusiones en este estudio es que la suplementación en GH puede actuar como un antioxidante porque nuestros resultados muestran en las ratas viejas que la GH activo enzimas endógenas antioxidantes (catalasa, glutation peroxidasa y glucosa-6-fosfato dehydrogenasa), disminuyen los daños oxidativos de los componentes celulares (proteína y ADN), y entonces se comporta como un antioxidante. Esto puede ayudar a explicar la protección contra la sarcopenia conferida por la suplementación con GH.

La síntesis de proteínas, mitochondriogenesis y prevención de la sarcopenia con GH

El mantenimiento de la masa muscular está regulada por el equilibrio entre la síntesis de proteínas y la proteólisis (Powers et al. 2011). La síntesis proteica muscular disminuye con la edad (Jones et al. 2009). El papel de p70S6K en la hipertrofia ha sido descrito previamente en varios modelos animales (Song et al. 2005). Una vez activada por la quinasa AKT, mTOR activa la iniciación de la traducción a través de la fosforilación de p70S6K que a su vez fosforila la proteína ribosómica S6 y permite la regulación de los ARNm que codifican el aparato de la traducción (Kimball et al. 2002). Se encontro una disminución significativa de la fosforilacion de Akt en el músculo esquelético de los animales viejos no tratados que ha sido completamente prevenida en las ratas tratadas con GH. Del mismo modo, la fosforilación de la p70S6K fue menor en los músculos de las ratas viejas que en las ratas jóvenes. Los animales viejos tratados con GH mostraron valores similares de fosfo-p70S6K a las de los animales jóvenes. Nuestros resultados están en contradicción con unos estudios previos que muestran que las inyecciones intraperitoneales de IGF-I aumentaron la fosforilación de p70S6K en animales jóvenes, pero no en los animales viejos (Li et al. 2003).

El debilitamiento de la capacidad hipertrófica muscular en los ancianos es debido en parte a potencial miogénico deterioro asociado con el envejecimiento (Marsh & Criswell 1997; Hansen et al. 2007). Por lo tanto, hemos tratado de comparar la respuesta miogénica del músculo de animales jóvenes a la de los animales tratados con GH. Myf-5 es un marcador bien conocido de la diferenciación de mioblastos y células satélite y facilita la reparación o la regeneración y el crecimiento de las fibras maduras (Kim et al. 2005). Se ha demostrado que el tratamiento con GH en el músculo aumenta la expresión del gen del IGF-I (Hameed et al.

2003) que está implicado en la activación de las células satélite (Goldspink & Harridge 2004). Nuestros resultados mostraron que, aunque el envejecimiento no ha dado lugar a una disminución del contenido proteico de Myf-5 en el músculo esquelético, la terapia de reemplazo de GH ha incrementado de manera significativa la cantidad de este factor miogénico.

Luego nos centramos en la miostatina un factor negativo de la regulación de la masa muscular (Goldspink & Harridge 2004). Pertenece a la familia de los TGF-ß, pero su expresión está restringida al tejido muscular (McPherron & Lee 1997). La ausencia o el bloqueo de la miostatina induce hipertrofia muscular masiva que se asignó inicialmente a la proliferación de las células satélites (Ten Broek et al. 2010). Sin embargo, se ha demostrado recientemente que la miostatina regula el equilibrio entre sintesis y degradacion proteica en las fibras musculares. Varios grupos de investigación han demostrado que la hipertrofia, en la ausencia de miostatina, implica poco o no las células satélites (Amthor et al. 2009; Welle et al. 2006). Las fibras hipertróficas no contienen más núcleos musculares o células satélites, y la miostatina no tiene ningún efecto significativo sobre la proliferación de células satélite (Amthor et al. 2009).

También encontramos un aumento de la expresión de p21 en el músculo de las ratas viejas (McKay y al. 2012), lo cual esta significativamente reducido por la terapia de sustitución de GH. Así, el efecto de la GH sobre estos dos factores puede contribuir a la prevención de la atrofia muscular. Datos muestran que el añadido de miostatina en medio de cultura de fibroblastos musculares induce un phosphorylation de la p38-MAPK (Li y al. 2008). p38 es una proteína quinasa que responde a una variedad de estímulos, incluyendo el estrés oxidativo y TNF-α (Derbré et al. 2012). Fue identificado como un probable mediador en la señalización catabólica dentro del músculo esquelético (Powers et al. 2007. Li et al. 2005). Por lo tanto, se midió la fosforilación de p38 en el músculo de nuestros animales. Como en el estudio de Williamson et al. (2003), encontramos un aumento significativo de la fosforilación de p38 en animales viejos, lo cual fue totalmente prevenido por la terapia de sustitución de GH.

Para identificar el mecanismo por lo cual la GH reduce la pérdida de la masa muscular durante el envejecimiento, medimos la expresión de dos ubiquitinas ligasas E3 específicas del músculo, MAFbx y MuRF1, bien conocidas para ser implicadas en la atrofia del músculo esquelético en varios modelos in vivo (Foletta et al. 2011). Aunque controvertidos (Edström et al. 2006), los niveles de ARNm de MuRF1 y de MAFbx incrementan significativamente en los músculos envejecidos (Clavel et al. 2006). En nuestro estudio, encontramos un aumento

significativo de contenido proteico de MuRF1 en el músculo de los animales viejos, lo cual fue prevenido por el tratamiento con GH. Sin embargo, no se encontraron cambios con MAFbx. Por lo tanto MuRF1 parece estar implicado en la sarcopenia relacionada con la edad.

El envejecimiento está al principio de una disminución del contenido y de la actividad mitocondrial (Miquel et al. 1980; Sastre et al. 1996). PGC-1α es un regulador mayor de la biogénesis mitocondrial (Puigserver et al. 1998; Viña et al. 2009) y responde muy rápidamente a los cambios de la homeostasis redox (St-Pierre et al. 2006; Viña et al. 2009; Gomez-Cabrera, Domenech & Viña 2008). Así como, lo vimos anteriormente, el envejecimiento aumenta los marcadores de estrés oxidativo en el músculo, y esto es prevenido por dosis relativamente bajas de GH. Por lo tanto, probamos si el envejecimiento provocaba una disminución de la expresión de PGC-1α en el músculo, y efectivamente fue el caso. El tratamiento con GH previno totalmente la disminución de PGC-1α y de su diana NRF-1 asociado con el envejecimiento. En la literatura, es descrito que PGC-1 α no se activa más de modo normal en respuesta al ejercicio en los animales viejos (Derbré et al. 2012). Esta falta de reactividad podría ser debida a una falta de GH porque la administración de esta última activo PGC-1α que tiene su vuelta acelera el mitochondriogénèse como lo demuestra el aumento del contenido proteico muscular de cytochrome C así como el aumento de la actividad de la citrato sintasa en los animales viejos tratados con GH. La GH podría activar PGC-1α vía el IGF-1 y la calcineurina como esto ha sido mostrado en el músculo cardíaco de ratas (Vescovo et al. 2005).

Por último, este estudio ofrece ciertas evidencias respecto a la efectividad de la restauración del perfil de de GH a preservar la masa muscular en los ancianos.

Estudio 2: La sobreexpresión de la glucosa-6-fosfato deshidrogenasa mejora la composición corporal y el rendimiento físico en ratones.

Estudio 3: Estado redox en condiciones de reposo y en respuesta a estímulos pro-oxidantes: impacto de la sobreexpresión de la glucosa-6-fosfato deshidrogenasa.

INTRODUCCIÓN

El mecanismo por lo cual la GH disminuye el estrés oxidativo es desconocido. Sin embargo la activación de la G6PDH por la GH que mostramos en el estudio precedente constituye una pista potencial. Por lo tanto, esto nos condujo a explorar la G6PDH, ya que ciertos trabajos mostraron que su surexpression en drosophila aumentaba la longevidad y protegía contra agresiones pro-oxidantes (Legan and al. 2008). Emitimos la hipótesis que la G6PDH podría entonces constituir una diana terapéutica alternativa a la GH de la cual conocemos los efectos deletéreos pocibles.

La G6PDH es la enzima limitante de la vía de los pentosas. Conduce particularmente a la síntesis de ribosa-5-fosfato, lo cual interviene en la síntesis de los ácidos nucleicos. La G6PDH utiliza como cofactor el NADP que se transforma en NADPH. El principal efecto antioxidante de la G6PDH pasa por este NADPH que contribuye a reducir la quantidad de glutation oxidado y activa la catalasa (Stanton 2012; Hecker & Leopold 2013).

El efecto antioxidante de la G6PDH ha sido bien demostrado. Así estudios *in vitro* mostraron que su inhibición devuelve las células más vulnerables al estrés oxidativo meintras su surexpression proteja contra el estrés oxidativo (Tian et al. 1998). Estos datos condujeron a investigar un papel eventual de la G6PDH sobre la longevidad, particularmente en drosophila. En esta especie, cuando la G6PDH se sobreexpressa, la longevidad espontánea es alargada muy significativamente así como la supervivencia cuando las drosophilas son expuestos a un riesgo prooxidante como la hiperoxia o el paracuat (Legan y al. 2008).

Por otra parte, trabajos sugieren que la G6PDH sería implicada en la regulación de la masa muscular. De hecho, varios casos clínicos de rabdomiolisis debidos a una deficiencia en G6PDH han sido observados, en los años 90 (Kimmick & Owen 1996). Por otro lado, numerosos estudios mostraron durante los años ochenta que la desregulación de su actividad era asociada con ciertas miopatías (Elias & Meijer 1983; Meijer & Elias 1984). También, ha sido mostrado que durante la regeneración muscular (conocida para implicar las células

satélites) la actividad de la G6PDH es considerablemente aumentada (Wagner et al. 1977; Wagner et al. 1978) mientras la síntesis de las proteínas y la síntesis de los ARN son aumentadas (Wagner et al. 1978). En el mismo sentido, dos estudios mostraron que la hipertrofia inducida por inyecciones de testosterona en ratas castradas o ratones sarcopenicos era asociada con un aumento de la expresión y\o de la actividad de la G6PDH en el músculo esquelético (Max 1984; Kovacheva et al. 2010).

Por lo tanto, todos estos datos nos condujeron a explorar sucesivamente el efecto de la sobreexpresión de la G6DPH en ratónes sobre la regulación del estatuto redox al reposo (medida de los daños oxidativos y de la expresión de enzimas antioxidantes al nivel sistémico y muscular), sobre la tolerancia al estrés oxidativo (en respuesta a un ejercicio exhaustivo y a la hiperoxia), sobre las capacidades físicas (consumo máximo de oxígeno, el tiempo de resistencia, fuerza de aggaramiento contra un dinamómetro y el tiempo de suspensión por las cuatro patas) y la composición corporal, particularmente la masa muscular (DEXA, peso de los músculos).

RESULTADOS/DISCUSIÓN

El estrés oxidativo en condiciones de reposo

La sobreexpresión de la G6PDH no altera el daño oxidativo sistémico, pero disminuye el daño oxidativo muscular

Numerosos estudios en roedores han demostrado que la reducción de la actividad y del contenido proteico de la G6PDH están asociados con un aumento del los daños oxidativos (Kumaran et al. 2004; Senthil Kumaran et al. 2008; Braga et al. 2008; Kovacheva et al. 2010). Para evaluar si la sobreexpresión de la G6PDH puede proteger contra el estrés oxidativo, se midió en condiciones de reposo, los daños oxidativos en el plasma (utilizado para evaluar el estrés oxidativo sistémico) y el músculo gastrocnemio.

Nuestros resultados mostraron que al nivel sistemico la oxidación de las proteínas y los niveles de peroxidación de lípidos no se redujeron por la sobreexpresión de la G6PDH. Esta falta de diferencia entre ratones WT y G6PDHtg confirman los datos de estos mismos marcadores en pacientes deficientes en G6PDH (Jamurtas et al. 2006; Theodorou et al. 2010).

En el músculo, la oxidación de las proteínas y la peroxidación lípidica fueron similares en ratones G6PDHtg en comparación con los ratones WT, lo que implica en nuestro modelo que el daño oxidativo muscular sería independiente de la G6PDH de estado. Sin embargo, estos resultados están contrarios a ciertos estudios que mostraron que en roedores envejecidos, una disminución de la actividad o del contenido proteico del G6PDH era asociada con un aumento del peroxydation de los lípidos y una disminución del cociente GSH/GSSG (Kumaran et al. 2004, 2008 ; Kovacheva et al. 2010). Estas divergencias podrían explicarse por la edad de nuestros animales. En efecto, estos estudios trabajaron con animales de edad entre 18 y 22 meses mientras que trabajamos entre animales de edad de 12-14 meses.

Sin embargo, por primera vez, este estudio también mostró que la sobreexpresión de G6PDH resultó en una disminución en el daño oxidativo del ADN total en el músculo gastrocnemio. De hecho, los valores de 8-hidroxi-2'-desoxiguanosina (8-OHdG) fue menor en los ratones G6PDHtg respecto a los ratones WT. Aunque esta relación nunca ha sido estudiada en el músculo esquelético, algunos autores han encontrado resultados similares en el cerebro. Así, Felix et al. (2002) y Jeng et al. (2013) encontraron valores más elevados de 8-OHdG y un aumento en el número de mutaciones del ADN en el cerebro de ratones deficientes de G6PDH en comparación con sus homólogos WT. El mecanismo por lo cual la G6PDH actuaría sobre el ADN no es conocido y deberia ser estudiado. Aunque esta relación no haya sido estudiada en nuestro estudio, nuestros ratones podrían presentar una disminución de las mutaciones del ADN en el músculo. Estos datos son completamente esencial porque numerosos estudios observaron en diferentes especies una acumulación de mutaciones del ADN mitocondrial en el músculo esquelético durante en el envejecimiento debido al aumento del estrés oxidativo (Lee et al. 1998; Bua et al. 2006; Figueiredo et al. 2009; Lee et al. 2010).

Estas mutaciones del ADN mitocondrial son la causa de defectos de la cadena de transporte de electrones y conducen a aberraciones morfológicas de las fibras musculares (Bua et al. 2006). Desde que se demostro que la G6PDH está presente en la mitocondria (Mailloux y Harper 2010), suponemos que los ratones que sobreexpresan la G6PDH están protegidos contra las mutaciones del ADN durante el envejecimiento.

El estrés oxidativo en respuesta a situaciones pro-oxidantes

Nuestros resultados muestran que los ratones G6PDHtg no estaban protegidos contra la hiperoxia. De hecho, el tiempo de supervivencia tras la exposición a hiperoxia era el mismo entre los ratónes G6PDHtg y sus homólogos WT. Del mismo modo, no se encontró ningún

efecto protector de la sobreexpresión de la G6PDH contra el daño muscular y el daño oxidativo inducido por el ejercicio exhaustivo. De hecho, encontramos aumentos similares en las concentraciones plasmáticas de creatina quinasa y lactato deshidrogenasa, así como proteínas oxidadas y 4-hidroxinonenal en los ratones WT y G6PDHtg en respuesta a un ejercicio exhaustivo. La falta de efecto protector contra el estrés oxidativo se podria explicar por el hecho de que la G6PDH proporciona tanto NADPH en sistemas antioxidantes y sistemas pro-oxidantes tales como la xantina oxidasa, la óxido nítrico sintasa y la NADPH oxidasa (Hecker & Leopold 2013) que están involucrados en la producción de radicales libres durante el ejercicio exhaustivo (Gomez-Cabrera et al. 2005; Gomez-Cabrera et al. 2010; Gomez-Cabrera et al. 2013). Esto también podría explicar la falta de diferencia en la exposición a hiperoxia.

La sobreexpresión de G6PDH mejora la composición corporal y el rendimiento físico

Con el fin de confirmar los datos que sugieren que G6PDH está implicado en la regulación de la masa muscular (Max 1984; Kovacheva et al. 2010), se realizó un estudio de la composición corporal. Se encontró que la sobreexpresión de G6PDH conduce a una disminución del peso corporal asociado con un incremento de la masa magra y una disminución de la masa adiposa. El aumento de la masa magra es debido a una mayor masa muscular porque la densidad mineral ósea, el peso de los órganos internos (corazón, hígado, riñones...) normalizados por el peso corporal fueron similares entre los ratones WT y G6PDHtg mientras que el peso de los músculos (gastrocnemio, tibial anterior, sóleo) normalizado por el peso corporal fueron mayores en los ratones G6PDHtg en comparación con los ratones WT. Además, el aumento del conteido proteíco de la cadena pesada miosina total del músculo en ratones G6PDHtg confirmó el aumento de masa muscula. La mejora de la composición corporal observada en ratones G6PDHtg se asoció con un mejor rendimiento físico que las observadas en los ratones WT. De hecho, encontramos que la fuerza muscular y el consumo máximo de oxígeno fueron más altos en los ratónes G6PDHtg. La mejora en la composición corporal, en particular el aumento de la masa muscular y el aumento de la fuerza muscular y el consumo máximo de oxígeno son de una importancia capital en la sarcopenia, que se caracteriza por una disminución en el peso y la fuerza muscular (Cruz-Jentoft et al. 2010). Además, una disminución de la masa grasa también tendría un efecto beneficioso sobre la función muscular. De hecho, durante la sarcopenia hay infiltraciones de grasa en el músculo que son predictivas de la discapacidad y la mortalidad (Visser et al. 2005).

La composición corporal puede ser influenciada por la actividad espontánea de los animales y/o el comportamiento de alimentación. Ambos parámetros fueron evaluados en ratones WT y G6PDHtg, y no mostraron ninguna diferencia entre los dos grupos de ratones, lo que nos permition descartar los dos fenómenos para explicar las diferencias en la composición corporal.

Para explicar este aumento de masa muscular en ratones G6PDHtg en comparación con WT, hemos investigado varias vías de señalización.

Nuestros resultados proporcionan pruebas de que la G6PDH juega un papel central en la regulación de la masa muscular, ya que esto se había sugerido previamente en varios estudios. De hecho, Max (1984) y Kovacheva et al. (2010) demostraron en varios modelos que la atrofia muscular es asociada con una disminución en la actividad y el contenido muscular de la G6PDH, mientras que la hipertrofia muscular se asocia con un aumento de ambos parámetros.

La regeneración muscular también es asociada con una actividad aumentada del G6PDH (Wagner y al. 1977; Wagner y al. 1978). Para explicar esto, fue propuesto que un aumento de la actividad de G6PDH conduciría a aumentar la síntesis ribose-5-fosfato (R5P) que conduciría a un aumento de la síntesis de ADN y de ARN, y en definitivo de proteínas. Esta hipótesis fue confirmada *in vitro*, donde la sobreexpresión de G6PDH resultó en un aumento en la velocidad de crecimiento de las células debido a una mayor síntesis de ADN que conduce a un aumento de la síntesis de proteínas (Tian et al. 1998, Kuo et al. 2000). Sin embargo, esto nunca ha sido confirmado *in vivo*. Pero en nuestro modelo animal, nuestros resultados mostraron un contenido en ADN total en el músculo gastrocnemio más alto en los ratones G6PDHtg en comparación con los ratones WT, que confirmó los estudios mencionados anteriormente. Además, las concentraciones de ácido úrico plasmáticos observados en ratones G6PDHtg son una prueba que la sobreexpresión de G6PDH aumenta el turnover de los ácidos nucleicos. Esto daria a los ratones G6PDGtg un capacidad mayor de síntesis de proteínas que podría aumentar su síntesis de proteínas, lo que explicaria el aumento de la masa muscular. Sin embargo, esto debe ser confirmado por medición directa del flujo de la síntesis de proteínas.

Por otra parte, estudiamos la vía de señalización de PI3K / Akt / mTOR con el fin de estudiar el impacto del surexpression de G6PDH sobre la traducción de las proteínas. Nuestros resultados (activación de Akt y P70S6K) no mostraron ninguna diferencia entre el ratón G6PDHtg y el WT.

En resumen, nuestro trabajo ha demostrado que la sobreexpresión de G6PDH en ratones disminuye significativamente el daño oxidativo del ADN en el músculo esquelético. Sin embargo, los mecanismos al principio de esta protección tienen que ser estudiados. Sorprendentemente, nuestros resultados mostraron una falta de protección de la sobreexpresión de la G6PDH contra el estrés oxidativo inducido por estímulos pro-oxidantes, debido probablemente al hecho que el NADPH sintetizado por la G6DPH esta involucrado en ambos sistemas pro y antioxidantes. Además, este trabajo es el primero en mostrar que los ratones que sobreexpresan la G6PDH mejora la composición corporal mediante la reducción de la masa grasa y el aumento de la masa muscular debido probablemente a un aumento en la capacidad de la síntesis de proteínas a través de una mayor contenido de ADN en el músculo. La mejora de la composición corporal se asocia con una mejora en la fuerza muscular y cualidades aeróbicas.

Finalmente, este trabajo muestra que la mejora de la actividad de G6PDH sería una buena estrategia para mejorar la composición corporal y el rendimiento físico. En un contexto más amplio, los efectos beneficiosos observados en respuesta a la sobreexpresión de G6PDH, conducirían a mejorar la salud de estos ratones y en última instancia aumentar la longevidad como se ha demostrado en un modelo de Drosophila que sobreexpresa G6PDH.

III) Conclusion

La esperanza de vida nunca ha sido tan larga en la historia de la humanidad. Sin embargo, esto conduce a un envejecimiento general de la población y, inevitablemente, a un aumento en la prevalencia de la sarcopenia, que a su vez contribuye al considerable aumento en el coste de la salud publica en nuestras sociedades. Para limitar este fenómeno, desarrollar estrategias eficaces para prevenir o tratar la sarcopenia es un gran desafío que requiere la comprensión de los mecanismos celulares y moleculares implicados en la aparición de la sarcopenia asi como los implicados en su prevención.

En esta tesis se ha tratado de responder a tres objetivos generales. El primer objetivo fue determinar *in vivo* cómo un estado redox pro-oxidante dentro del tejido muscular envejecido podría modular las vías de señalización implicadas en los mecanismos moleculares de la sarcopenia. El segundo objetivo era mostrar que el retorno al funcionamiento normal de estas vías de señalización requiere una restauración de la homeostasis redox. Por último, el tercer objetivo de esta tesis fue identificar los posibles mecanismos moleculares implicados en el mantenimiento y/o restauración de la homeostasis redox.

En un primer estudio realizado en ratas viejas, comprobamos que el estrés oxidativo debido al envejecimiento conducía a una alteración de la vía PI3K/Akt/mTOR que sugería una disminución de la síntesis de las proteínas mientras que al mismo tiempo un aumento de la expresión de MuRF1 y MAFbx sugería un aumento del proteolisis por el sistema ubiquitina-proteasoma. Además, una disminución de la función mitocondrial y de la mitocondriogénesis ha sido encontrada.

En un segundo paso, hemos demostrado que la terapia de reemplazo con hormona de crecimiento en ratas vieja prevenia la sarcopenia a través de efectos antioxidantes, anabólicos y anti catabólicos. En este primer estudio, se encontró que la G6PDH podría ser un posible candidato para explicar el efecto antioxidante de la hormona del crecimiento.

En los estudios 2 y 3, se encontró que los ratones transgénicos que sobreexpresan la G6PDH han mejorado su composición corporal que se caracteriza por un menor peso corporal, una reducción de grasa corporal y un aumento de la masa muscular. Además, se observó que los ratones G6PDHtg presentan mejores cualidades aeróbica y fuerza muscular. Además, observamos una disminución en el daño oxidativo del ADN en los ratónes

G6PDHtg. Aunque, sorprendentemente, no hemos encontrado ningún efecto protector de la sobreexpresión contra el estrés oxidativo inducido por el ejercicio exhaustivo, un efecto perjudicial también estuvo ausente.

Aunque los mecanismos que podrían explicar los efectos beneficiosos de la sobreexpresión de la G6DPH estén todavía a descubrir, nuestros resultados abren una puerta al desarrollo de estrategias de lucha contra la sarcopenia, pero más en general para mejorar la composición corporal y el rendimiento físico basada sobre la activación de la G6PDH. Sin embargo, antes de desarrollar este tipo de estrategias, son necesarios más estudios para asegurarse de que no hay peligro de activar la G6DPH.

RÉSUMÉ EN FRANÇAIS

I) Introducción

Il y a environ 80 ans, MacDonald Critchley fut le premier à reconnaitre que la masse musculaire diminue au cours du vieillissement et remarqua que cette perte était d'autant plus importante dans les muscles des membres inférieurs et supérieurs (Critchley 1931). Près de soixante ans plus tard, en 1988, au cours d'un congrès à Albuquerque (Etats-Unis) portant sur l'évaluation de l'état de santé des personnes âgées, Rosenberg fit remarqué que «aucune baisse avec l'âge n'est plus dramatique ou potentiellement plus importante fonctionnellement que la diminution de la masse musculaire ». Il souligna que pour faire reconnaitre ce phénomène par la communauté scientifique, celui-ci nécessitait un nom et proposa le terme «sarcopénie» (du grec «sarx» : chair + « penia » : perte). Par la suite, la sarcopénie fut définie comme la diminution générale et progressive de la masse musculaire qui survient avec l'âge (Roubenoff & Hughes 2000). Cependant, cette définition ne fut pas été acceptée par l'ensemble des cliniciens et des chercheurs et a beaucoup évolué. Finalement, le consensus actuel définit la sarcopénie comme «un syndrome gériatrique d'abord caractérisé par une diminution de la masse musculaire qui en s'aggravant participera à une diminution de la force musculaire et à une détérioration des performances physique» (Muscaritoli et al. 2010; Cruz-Jentoft et al. 2010; Fielding et al. 2011; Morley et al. 2011).

Grâce aux progrès sociaux, technologiques et médicaux, l'espérance de vie n'a cessé d'augmenter depuis le 19ème siècle dans nos sociétés occidentales modernes, conduisant à un vieillissement général de la population. Actuellement, mondialement, il est prévu que le nombre de personnes âgées double d'ici 2050, passant de 11% de la population à 22% (ONU 2007). Inévitablement, en raison de ce vieillissement de la population, la prévalence de la sarcopénie est en croissance, et actuellement on estime que entre un quart et la moitié des hommes et des femmes âgés de 65 ans et plus, sont susceptibles d'être sarcopéniques (Janssen 2004). Les conséquences de la prévalence croissante de la sarcopénie sont généralement considérés comme catastrophiques sur les coûts de santé publique. Ainsi, le coût total de la sarcopénie au système de santé américain a été estimé à environ 18,4 milliards de dollars (Janssen et al. 2004). A l'avenir, du fait que les personnes âgées de plus de 69 ans représentent la tranche de la population américaine qui croît le plus rapidement, il semblerait qu'à l'avenir ce coût ne fasse qu'augmenter (Manton et Vaupel, 1995). Ces dépenses de santé sont liés à une détérioration générale de l'état physique conduisant à un risque accru de chutes, une incapacité progressive à accomplir des activités élémentaires de la vie quotidienne et la perte d'autonomie des personnes âgées (Goodpaster et al. 2006; Delmonico et al. 2007).

Cependant, plusieurs stratégies sont reconnues comme efficaces pour prévenir, retarder ou traiter la sarcopénie. Ainsi, développer des thérapies n'aiderait pas seulement à améliorer la qualité de vie des personnes sarcopéniques, mais aussi réduirait les coûts économiques associés à la sarcopénie, ce qui serait bénéfique à la société dans son ensemble. Actuellement, l'exercice physique est indéniablement la stratégie plus efficace dans la lutte contre la sarcopénie, car il peut conduire à augmenter la masse musculaire, la force et les performances physiques (Pillard et al. 2011; Di Luigi et al. 2012; Wang & Bai 2012; Montero & Serra 2013). Cependant, la mise en œuvre à grande échelle d'une telle intervention est entravée par le manque de motivation de la plupart des personnes. En outre, de nombreuses personnes âgées sont non ambulatoires ou ont des comorbidités telles que de l'arthrose modérée à sévère (Bennell & Hinman 2011) ou certaines formes de maladies cardiovasculaires instables ne permettant pas leur participation à des protocoles d'exercices physiques (Williams et al. 2007). Pour surmonter ces obstacles, le développement de thérapies alternatives telles que les stratégies antioxydantes et des thérapies de remplacement hormonales (testostérone et hormone de croissance) semblent nécessaires.

Le muscle squelettique est un organe qui a des propriétés spécifiques lui conférant un rôle central dans la locomotion, l'exécution des activités de la vie quotidienne, le maintien de la posture et de l'équilibre. Afin d'assurer ces fonctions essentielles, le muscle doit avoir une masse suffisante que chacun doit chercher à préserver. Comme décrit précédemment, certaines des conséquences les plus graves du vieillissement sont ses effets sur les muscles squelettiques en particulier la perte progressive de la masse et de la fonction qui ont une incidence sur la qualité de la vie, et, finalement, sur l'espérance de vie (Cruz-Jentoft 2012).

A l'heure actuelle, les mécanismes à l'origine de la sarcopénie ne sont pas encore bien définis, et par conséquent, ils font encore le sujet de nombreux travaux de recherche. Toutefois, un turnover protéique négatif (Combaret et al. 2009), une altération des dynamiques mitochondriales (Calvani et al. 2013), une diminution des capacités de régénération musculaires (Snijders et al. 2009; Hikida 2011), ainsi que l'exacerbation de l'apoptose des noyaux musculaires (Marzetti et al. 2012) sont généralement considérés comme des mécanismes cellulaires impliqués dans l'atrophie musculaire conduisant à la sarcopénie. Ces mécanismes sont eux-mêmes dépendant d'une multitude de facteurs systémiques et cellulaires tels que la diminution de la production d'hormones anabolisantes (GH, l'IGF-1, la testostérone, l'insuline). Les liens et les interactions entre ces sécrétions d'hormones diminuées et les dysfonctionnements cellulaires cités précédemment restent en partie inconnus. Un candidat potentiel pourrait être le stress oxydant chronique liée à l'âge, dont des

études récentes ont souligné son implication dans la sarcopénie (Semba et al. 2007; Safdar et al. 2010).

Ainsi, le muscle sarcopénique présente une surproduction d'espèces radicalaires ou non radicalaires dérivées de l'oxygène et de l'azote (RONS) (Capel et al. 2004; Capel, Rimbert, et al. 2005; Capel, Demaison, et al. 2005; Chabi et al. 2008; Jackson et al. 2011; Andersson et al. 2011; Miller et al. 2012). Cette surproduction de RONS est principalement due à des dysfonctions mitochondriales (Capel, Rimbert, et al. 2005; Chabi et al. 2008) et une augmentation de l'activité de la xanthine oxydase (Lambertucci et al. 2007; Ryan et al. 2011), et conduit à une augmentation des dommages oxydatifs des différents composant cellulaires et moléculaires du muscle squelettique. Ces dommages oxydatifs reflètent l'incapacité des systèmes antioxydants à prendre en charge la surproduction de RONS et attestent du déséquilibre de la balance « oxydants-pro oxydants » conduisant à une altération de l'homéostasie redox (Jones 2006). Il semblerait que la restauration de l'homéostasie redox par certaines des stratégies de lutte contre la sarcopénie implique une augmentation du contenu protéique et/ou de l'activité de l'enzyme glucose-6-phosphate déshydrogénase (G6PDH) au sein du muscle (Kovacheva et al. 2010; Sinha-Hikim et al. 2013). La G6PDH est l'enzyme limitante de la voie des pentoses phosphates connue pour être la source de NADPH de certains systèmes antioxydants (Scott et al. 1993). De plus, quelques données *in vitro* et *in vivo* suggèrent que la G6DPH jouerait un rôle important dans la régulation de la masse musculaire. Cependant, ces données restent à confirmer.

Objectifs:

Dans ce contexte, cette thèse va tenter de répondre à trois objectifs généraux. Le premier objectif est de déterminer *in vivo* dans quelles mesures un état redox pro-oxydant dû au vieillissement dans le tissu musculaire peut moduler les voies de signalisation impliquées dans les mécanismes moléculaires à l'origine de la sarcopénie. Le deuxième objectif est de montrer que le retour à un fonctionnement normal de ces voies de signalisation est associé à une restauration de l'homéostasie redox. Finalement, le troisième objectif de cette thèse est d'identifier des acteurs et les possibles mécanismes par lesquels l'homéostasie redox pourrait être maintenue.

Les objectifs spécifiques sont les suivants:

- Déterminer si le statut pro-oxydant chronique dans le muscle squelettique de rats âgés peut moduler les voies de signalisations conduisant à la sarcopénie impliquées dans la synthèse des protéines et la protéolyse, mais aussi dans la régénération musculaire et la mitochondriogenèse. Nous émettons l'hypothèse que le stress oxydatif conduirait à une down-régulation des voies de signalisation et PI3K/Akt/mTOR PGC-1α/Tfam/Nrf-1, et à une up-régulation des marqueurs du système ubiquitine protéasome ainsi que des inhibiteurs de la régénération musculaire (étude 1).

- Déterminer dans quelles mesures et par quels mécanismes un traitement substitutif à l'hormone de croissance permet de prévenir la sarcopénie chez des rats âgés. Nous faisons deux hypothèses.

    3) La GH par l'intermédiaire d'une augmentation des concentrations d'IGF-1 circulant permettrait la restauration d'un fonctionnement normal de la voie de signalisation PI3K/Akt/mTOR tout en diminuant l'expression de plusieurs acteurs du système ubiquitine-protéasome dépendant et d'inhibiteurs de la régénération musculaire. Un possible effet sur la mitochondriogenèse est également envisagé (étude 1).

4) Ces effets bénéfiques seraient associés à une amélioration de l'état redox en particulier grâce à la up-régulation de certaines enzymes antioxydants (étude 1).

- Déterminer *in vivo* chez un modèle de souris surexprimant la glucose-6-phosphate déshydrogénase, les rôles de cette enzyme dans la régulation de la de la composition corporelle (masse musculaire et masse adipeuse) et ses impacts sur les performances physiques (force musculaire, consommation maximale d'oxygène et endurance) (étude 2).

- Déterminer *in vivo* si la surexpression de la G6PDH permet d'améliorer au repos le statut redox et de protéger dans des situations pro-oxydantes (exercice exhaustif et hyperoxie) (étude 3).

II) Contribution personnelle

<u>Etude 1:</u> La thérapie de remplacement à l'hormone de croissance prévient la sarcopénie par un double mécanisme: l'amélioration du turnover protéique et des défenses antioxydantes.

Étude publiée dans *The Journals of Gerontology Series A Biological Sciences*

Growth hormone replacement therapy prevents sarcopenia by a dual mechanism: improvement of protein balance and of antioxidant defenses

Authors: Brioche T[1,2], Kireev RA[3], Cuesta S[3],Gratas-Delamarche A[2], Tresguerres JA[3], Gomez-Cabrera MC[1], and Viña J[1]

Affiliations:[1]Department of Physiology. University of Valencia. Fundacion Investigacion Hospital Clinico Universitario/INCLIVA. Spain.

[2]Laboratory "Movement Sport and health Sciences", University Rennes. France

[3]Department of Physiology. University Complutense of Madrid. Spain

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Address correspondence to:

Jose Viña, Professor, MD (Corresponding author)

Department of Physiology. Faculty of Medicine. University of Valencia.

Av. Blasco Ibañez, 15, Valencia

46010 Spain

Phone: (34) 96 386 46 50

Fax: (34) 96 386 46 42

Email: jose.vina@uv.es

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INTRODUCTION

Le consensus actuel définit la sarcopénie comme un syndrome gériatrique d'abord caractérisée par une diminution de la masse musculaire qui en s'aggravant participera à une diminution de la force musculaire et une détérioration des performances physiques» (Muscaritoli et al. 2010; Cruz-Jentoft et al. 2010; Fielding et. al 2011; Morley et al. 2011) aboutissant à l'apparition de nombreuses comorbidités tels que un handicap physique, une altération de la qualité de vie et une réduction de l'espérance de vie (Evans 1995). Cette perte de masse musculaire se produit à un taux de 3 à 8% par décennie après l'âge de trente ans et ce taux augmente après soixante ans (Holloszy 2000). De récentes estimations montrent que entre un quart et la moitié des hommes et des femmes âgés de plus de soixante-cinq ans sont considérés comme sarcopéniques (Janssen 2004). La sarcopénie joue un rôle majeur dans le développement du syndrome de fragilité, et progressivement augmente le risque de chutes et diminue les capacités des personnes à réaliser les activités de la vie quotidienne (Evans 1995). Finalement, les sujets atteints de sarcopénie dans les stades les plus avancés perdent leur indépendance et finissent par être institutionnalisés (Wolfe 2006).

Certaines hormones sont connues pour avoir un effet sur la masse, la force et la fonction musculaire (Cruz-Jentoft 2012). Parmi celles-ci, l'hormone de croissance (GH) est une des plus étudiées (Cruz-Jentoft 2012). Les niveaux de GH sont habituellement réduits chez les sujets âgés ainsi que l'amplitude et la fréquence de sa sécrétion pulsatile (Cruz-Jentoft 2012). Ainsi, il a été émis l'hypothèse que la GH serait efficace dans la prévention de la perte de la masse musculaire au cours du vieillissement (Giannoulis et al. 2012).

Dans notre étude, nous avons cherché à élucider le rôle d'une thérapie de remplacement à la GH chez des rats âgés en comparant des animaux âgés (24 mois) à des animaux jeunes (3 mois) et à des animaux âgés traités avec de la GH (8 semaines, 2 mg/kg répartis en 2 injections sous-cutanées quotidiennes). Nous nous sommes centrés sur quatre des principaux mécanismes impliqués dans l'apparition et la progression de la sarcopénie: l'altération de la biogenèse mitochondriale, l'augmentation du stress oxydatif, l'augmentation de la dégradation des protéines et la dimiution de leur synthèse (Doherty 2003; Derbré et al. 2012).

Dans cette étude, nous présentons des éléments montrant que la restauration du profil de la GH est une bonne intervention pour améliorer ou préserver la masse musculaire squelettique chez des animaux âgés.

RESULTATS/DISCUSSION

Effet du vieillissement et de la thérapie de substitution de GH sur la composition corporelle des rats

Malgré le grand nombre d'études visant à évaluer les effets de la supplémentation en GH sur la masse musculaire, les résultats controversés rapportés dans la littérature maintiennent le débat quant à savoir si la GH peut ou ne peut être utilisée pour lutter contre la sarcopénie (von Haehling et al. 2012). Les résultats contrastés rapportés peuvent s'expliquer par les différences méthodologiques telles que les doses utilisées. Des doses élevées de GH sont fréquemment la cause d'effets indésirables (Papadakis et al. 1996; Holloway et al. 1994). C'est pourquoi dans notre étude, nous avons utilisé des doses relativement faibles. Nos résultats ont montré que les concentrations plasmatiques d'IGF-1 étaient plus faibles chez les animaux âgés que chez les jeunes animaux mais le traitement à la GH a permis de rétablir des valeurs comparables à celles des animaux jeunes.

Dans notre cas, la thérapie de substitution de GH est efficace pour prévenir la perte de masse musculaire liée à l'âge. Au cours de l'étude, nous avons constaté que les rats jeunes ont augmenté leur poids alors que les rats âgés en ont perdu. Cependant, les animaux âgés traités avec la GH ont montré une augmentation du poids, sensiblement différente de la perte de poids survenue chez les rats âgés non traités. Cette perte est principalement due à l'évolution de la masse maigre parce que l'index spécifique de gravité (SGI : index calculé à partir d'une technique de pesée hydrostatique) est passée de 5 chez les animaux jeunes à 3 chez les animaux âgés. Le SGI est un indice qui évalue la masse maigre et la masse adipeuse; plus il est élevé, plus la masse maigre de l'animal est élevée. Nos données montrent également que l'administration de GH augmente considérablement le SGI des rats âgés, ce qui signifie que la GH, par ses propriétés anabolisantes, anti-adipogènes et lipolytiques, est capable d'augmenter la masse musculaire et de réduire la masse adipeuse (Castillo et al. 2004; Castillo et al. 2005).

Nous avons également évalué l'atrophie du muscle gastrocnémien en pesant les muscles, et nous avons constaté une diminution de 30% du poids des muscles chez les animaux âgés comparés aux animaux jeunes. Cette atrophie a été complètement prévenue chez les animaux âgés traités à la GH.

L'effet antioxydant de la thérapie de substitution de GH

La théorie du vieillissement des radicaux libres a fourni une base théorique pour concevoir des expériences pour comprendre le vieillissement (Gomez-Cabrera et al. 2012). Il est maintenant bien établi que la up-régulation des défenses antioxydantes endogènes est un mécanisme efficace pour prévenir les dommages oxydatifs associés à une production excessive de radicaux libres (Gomez-Cabrera, Domenech & Viña 2008; Gomez-Cabrera, Domenech, Romagnoli, et al. 2008). Les effets de la GH sur la sarcopénie ont été largement étudiés (Brill et al. 2002; Papadakis et al. 1996), mais jusqu'à présent, ils n'ont jamais été centré sur la prévention des dommages des radicaux libres. Une des principales conclusions rapportées dans cette étude est que la supplémentation en GH peut agir comme un antioxydant parce que nos résultats montrent chez les rats âgés que la GH active des enzymes endogènes antioxydantes (catalase, glutathion peroxydase et glucose-6-phosphate déshydrogénase), diminuent les dommages oxydatifs des composants cellulaires (protéine et ADN), et donc se comporte comme un antioxydant. Ceci peut contribuer à expliquer la protection contre la sarcopénie conféré par la supplémentation en GH.

Synthèse protéique, mitochondriogénèse et prévention de la sarcopénie par la GH

Le maintien de la masse musculaire est régulé par la balance entre la synthèse protéique et la protéolyse (Powers et al. 2011). La synthèse protéique musculaire diminue avec l'âge (Jones et al. 2009). L'implication de p70S6K dans l'hypertrophie a été rapportée chez divers modèles animaux (Song et al. 2005). Une fois activé par la kinase AKT, mTOR active l'initiation de la traduction via la phosphorylation de p70S6K qui à son tour phosphoryle la protéine ribosomale S6 et permet la up-régulation d'ARNm encodant l'appareil traductionnel (Kimball et al. 2002). Nous avons constaté une diminution significative de la phosphorylation de Akt dans le muscle squelettique des animaux âgés non traités qui a été complètement prévenue chez ceux traités à la GH. De même, la phosphorylation de p70S6K était plus faible dans les muscles des rats âgés que dans ceux des rats jeunes. Les animaux âgés traités avec la GH ont montré des valeurs phospho-p70S6K similaires à celle des animaux jeunes. Nos résultats sont en contradiction avec de précédentes études montrant que des injections intra-péritonéales d'IGF-I augmentent la phosphorylation de p70S6K chez des animaux jeunes mais pas chez des animaux âgés (Li et al. 2003).

L'affaiblissement de la capacité hypertrophique musculaire chez les sujets âgés est due en partie à une altération du potentiel myogénique lié au vieillissement (Marsh & Criswell

1997; Hansen et al. 2007). Ainsi, nous avons cherché à comparer la réponse myogénique du muscle d'animaux jeunes à celui d'animaux âgés traités à la GH. Myf-5 est un marqueur bien connu de la différenciation des myoblastes et des cellules satellite et facilite la réparation ou la régénération et la croissance des myofibres matures (Kim et al. 2005). Il a été montré que le traitement GH dans le muscle augmente l'expression du gène de l'IGF-I (Hameed et al. 2003) qui est impliqué dans l'activation des cellules satellites (Goldspink & Harridge 2004). Nos résultats ont montré que, bien que le vieillissement n'ait pas entrainé une diminution du contenu protéique de myf-5 dans le muscle squelettique, la thérapie de substitution de GH a augmenté de manière significative les quantités de ce facteur myogénique.

Nous nous sommes ensuite centré sur la myostatine, un facteur négatif de la régulation de la masse musculaire (Goldspink & Harridge 2004). Celui-ci appartient à la famille des TGF-ß, mais son expression est restreinte au tissu musculaire (McPherron & Lee 1997). L'absence ou le blocage de la myostatine induit une hypertrophie musculaire massive qui initialement fut attribué à la prolifération des cellules satellites (Ten Broek et al. 2010). Cependant, il a été récemment montré que la myostatine régule la balance protéique dans les fibres musculaires. Plusieurs groupes de recherche ont montré que l'hypertrophie, en l'absence de la myostatine, implique peu ou pas les cellules satellites (Amthor et al. 2009; Welle et al. 2006). Les fibres hypertrophiques ne contiennent pas plus de noyaux musculaires ou de cellules satellites et la myostatine n'a pas d'effet significatif sur la prolifération des cellules satellites (Amthor et al. 2009). Nous avons aussi constaté une augmentation de l'expression de p21 dans le muscle des rats âgés (McKay et al. 2012) qui est significativement réduite par la thérapie de substitution de GH. Ainsi, l'effet de la GH sur ces deux facteurs peut contribuer à la prévention de l'atrophie musculaire. Des données montrent que l'ajout de myostatine au milieu de culture de fibroblastes musculaires induit une phosphorylation de la p38-MAPK (Li et al. 2008). p38 est une protéine kinase qui répond à une variété de stimuli, y compris le stress oxydatif et du TNF-α (Derbre et al. 2012), et a été identifiée comme un médiateur probable dans la signalisation catabolique au sein du muscle squelettique (Powers et al. 2007; Li et al. 2005). Ainsi, nous avons mesuré la phosphorylation de p38 dans le muscle de nos animaux. Comme dans l'étude de Williamson et al. (2003), nous avons trouvé une augmentation significative de la phosphorylation de p38 chez les animaux âgés qui a été prévenue par la thérapie de remplacement de GH.

Pour identifier le mécanisme par lequel GH réduit la perte de la masse musculaire au cours du vieillissement, nous avons mesuré l'expression des deux ubiquitines ligases E3 spécifiques du muscle MAFbx et MuRF1, bien connues pour être impliquées dans l'atrophie

du muscle squelettique dans plusieurs modèles *in vivo* (Foletta et al. 2011). Bien que controversés (Edström et al. 2006), les niveaux d' ARNm de MuRF1 et MAFbx sont nettement augmenté dans les muscles âgés (Clavel et al. 2006). Nous avons constaté une augmentation significative du contenue musculaires de MuRF1 chez les animaux âgés qui a été prévenu par le traitement à la GH. Cependant, nous n'avons pas trouvé de changement concernant MAFbx. Ainsi, MuRF1 semble être impliquée dans la sarcopénie liée à l'âge.

Le vieillissement est à l'origine d'une diminution du contenu et de l'activité mitochondrial (Miquel et al. 1980; Sastre et al. 1996). PGC-1α est un régulateur majeur de la biogénèse mitochondriale (Puigserver et al. 1998; Viña et al. 2009) et répond très rapidement aux changements de l'homéostasie rédox (St-Pierre et al. 2006; Viña et al. 2009; Gomez-Cabrera, Domenech & Viña 2008). Comme, nous l'avons vu précédemment, le vieillissement augmente les marqueurs de stress oxydatif dans le muscle, et ceci est prévenu par des doses relativement faibles de GH. Nous avons donc testé si le vieillissement entrainait une diminution de l'expression de PGC-1α dans muscle et ce fut effectivement le cas. Le traitement à GH prévient totalement la diminution de PGC-1α et de sa cible NRF-1 associée au vieillissement. Dans la littérature, il est décrit que PGC-1α ne s'active plus de façon normale en réponse à l'exercice chez des animaux âgés (Derbré et al. 2012). Ce manque de réactivité pourrait être dû à un manque GH car l'administration de cette dernière active PGC-1α qui a son tour active la mitochondriogénèse comme en témoigne l'augmentation du contenu protéique musculaire du cytochrome C ainsi que l'augmentation de l'activité de la citrate synthase chez les animaux âgés traités à la GH. La GH pourrait activer PGC-1α via l'IGF-1 et la calcineurine comme ceci a été rapporté dans le muscle cardiaque chez le rat (Vescovo et al. 2005).

Finalement, cette étude apporte un certain nombre de preuves concernant l'efficacité de la restauration de profil de la GH pour préserver la masse musculaire chez les personnes âgées.

<u>Etude 2:</u> La surexpression de glucose-6-phosphate déshydrogénase améliore la composition corporelle et les performances physiques chez la souris.

<u>Etude 3:</u> Statut redox dans des conditions de repos et en réponse à des stimuli pro-oxydants: impact de la surexpression de la glucose-6-phosphate déshydrogénase.

INTRODUCTION

Le mécanisme par lequel la GH diminue le stress oxydant est inconnu. Cependant l'activation de la G6PDH par la GH que nous avons montré dans l'étude précédente constitue une piste potentielle. Ceci nous a donc conduit à explorer la G6PDH, d'autant plus que certains travaux ont montré que sa surexpression chez la drosophile augmente la longévité et protège contre des agressions pro-oxidantes (Legan et al. 2008). Nous avons émis l'hypothèse que la G6DPH pourrait alors constituer une cible thérapeutique en alternative à la GH dont on connait les effets délétères.

La G6PD est l'enzyme limitante de la voie des pentoses. Elle conduit notamment à la synthèse d'un C5 le ribose 5P qui intervient dans la synthèse des acides nucléiques. Elle utilise comme cofacteur le NADP qu'elle transforme en NADPH. L'effet antioxydant majeur de la G6PDH passe par ce NADPH qui contribue à réduire le taux de glutathion oxydé et active la catalase (Stanton 2012; Hecker & Leopold 2013).

L'effet antioxydant de la G6PDH a bien été démontré. Ainsi des études *in vitro* ont montré que son inhibition rends les cellules plus vulnérables au stress oxydant alors que sa surexpression protège contre le stress oxydant (Tian et al. 1998). Ces données ont conduit à rechercher un éventuel effet de la G6PDH sur la longévité, notamment chez la drosophile. Dans cette espèce, lorsque la G6PDH est surexprimée, la longévité spontanée est allongée très significativement ainsi que la survie lorsque les drosophiles sont exposées à un risque pro-oxydant majeur comme l'hyperoxie ou le paracuat (Legan et al. 2008).

D'autre part, des travaux suggèrent que la G6PDH serait impliqué dans la régulation de la masse musculaire. En effet, plusieurs cas cliniques de rhabdomyolyse due à une déficience en G6PDH ont été rapportés, dans les années 90 (Kimmick & Owen 1996). Par ailleurs, de nombreuses études ont montré depuis les années quatre-vingt que la dérégulation de son activité est associée à certaines myopathies (Elias & Meijer 1983; Meijer & Elias

1984). Aussi, il a été montré que lors de la régénération musculaire (connue pour impliquer les cellules satellites) l'activité de la G6PDH est considérablement augmentée (Wagner et al. 1977; Wagner et al. 1978) tandis que la synthèse des protéines et la synthèse d'ARN sont augmentées (Wagner et al. 1978). Dans le même sens, deux études ont montré que l'hypertrophie induite par des injections de testostérone chez des rats castrés ou des souris sarcopénique est associée à une augmentation de l'expression et/ou de l'activité de la G6PDH dans le muscle squelettique (Max 1984; Kovacheva et al. 2010).

Toutes ces données, nous ont donc conduit à explorer successivement l'effet de la surexpression de la G6PDH chez la souris sur la régulation du statut redox au repos (mesure des dommages oxydatifs et de l'expression d'enzymes antioxydantes au niveau systémique et musculaire), sur la tolérance au stress oxydant (en réponse à un exercice exhaustif et à l'hyperoxie), sur les capacités physiques (consommation maximale d'oxygène, temps d'endurance, force d'agrippement contre dynamomètre et temps de suspension par les quatres pattes) et la composition corporelle) et notamment la masse musculaire (DEXA, pesée des muscles).

RESULTATS/DISCUSSION

Le stress oxydatif dans des conditions de repos

La surexpression de G6PDH ne modifie pas les dommages oxydatifs systémiques mais diminue les dommages oxydatifs musculaires

De nombreuses études chez les rongeurs ont montré que la diminution de l'activité et du contenue protéique de la G6PDH sont associés à une augmentation des dommages oxydatifs (Kumaran et al. 2004; Senthil Kumaran et al. 2008; Braga et al. 2008; Kovacheva et al. 2010). Afin d'évaluer si la surexpression G6PDH pourrait protéger contre le stress oxydatif, nous avons mesuré dans des conditions de repos dommages oxydatifs dans le plasma (utilisés pour évaluer le stress oxydant systémique) et dans le muscle gastrocnémien.

Nos résultats ont montré au niveau systémique que l'oxydation des protéines et la peroxydation lipidique n'ont pas été réduits par la surexpression de la G6PDH. Cette absence de différence entre les souris WT et G6PDHtg renforce indirectement les données concernant ces mêmes marqueurs chez des patients déficients en G6PDH (Jamurtas et al. 2006; Theodorou et al. 2010).

Dans le muscle, l'oxydation des protéines et de la peroxydation lipidique était similaire chez les souris G6PDHtg par rapport aux souris WT, ce qui suppose dans notre modèle que les dommages oxydatifs musculaires seraient indépendants du statut en G6PDH. Cependant, ces résultats sont en contradiction avec certaines études qui ont montré que chez des rongeurs âgés, une diminution de l'activité ou du contenu protéique de la G6PDH est associée à une augmentation de la peroxydation des lipides et une diminution du rapport GSH/GSSG (Kumaran et al. 2004, 2008 ; Kovacheva et al. 2010). Ces divergences pourraient s'expliquer par l'âge de nos animaux. En effet, ces études ont travaillé avec des animaux âgés entre 18 et 22 mois alors que nous avons travaillé chez des animaux âgés de 12-14 mois.

Cependant, pour la première fois, cette étude a également montré que la surexpression de la G6PDH a conduit à une diminution des dommages oxydatifs de l'ADN total dans le muscle gastrocnémien. En effet, les valeurs de 8-hydroxy-2'-deoxyguanosine (8-OHdG) étaient inférieure chez les souris G6PDHtg par rapport aux souris WT. Bien que cette relation n'ait jamais été étudiée dans le muscle squelettique, certains auteurs ont trouvé des résultats concordants dans le cerveau. Ainsi, Felix et al. (2002) et Jeng et al. (2013) ont trouvé des valeurs plus élevées de 8-OHdG et une augmentation du nombre de mutation de l'ADN dans le cerveau de souris déficientes en G6PDH par rapport à leurs congénères WT. Le mécanisme par lequel la G6PDH agirait sur l'ADN n'est pas connu et doit être étudiée. Bien que cette relation n'a pas été étudié dans notre étude, nos souris pourraient présenter une diminution des auraient mutations de l'ADN dans le muscle. Ces données sont tout à fait essentiel car de nombreuses études ont observés dans différentes espèces une accumulation de mutations de l'ADN mitochondrial dans le muscle squelettique liée à l'âge en raison du stress oxydant (Lee et al. 1998; Bua et al. 2006; Figueiredo et al. 2009; Lee et al. 2010). Ces mutations de l'ADN mitochondrial sont à l'origine d'anomalies de la chaine de transport des électrons et conduisent à des aberrations morphologiques des fibres musculaires (Bua et al. 2006). Depuis, qu'il a été montré que la G6PDH est présente dans les mitochondries (Mailloux & Harper 2010), nous supposons que les souris surexprimant la G6PDH seraient protégées contre les mutations de l'ADN au cours du vieillissement.

Stress oxydant en réponse à des situations pro-oxydantes

Nos résultats montrent que les souris G6PDHtg n'étaient pas protégées contre l'hyperoxie. En effet, le temps de survie lors de l'exposition à l'hyperoxie était le même entre

les souris G6PDHtg et leurs homologues de WT. De la même manière, nous n'avons pas trouvé d'effet protecteur de la surexpression de la G6PDH contre les dommages musculaires et les dommages oxydatifs dans le muscle induits par un exercice exhaustif. En effet, nous avons constaté des augmentations similaires des concentrations plasmatiques de créatine kinase et de lactate déshydrogénase ainsi que de protéines oxydées et de 4-hydroxynonénal chez la souris G6PDHtg et WT en réponse à un exercice exhaustif. L'absence d'effet protecteur contre le stress oxydant pourrait s'expliquer par le fait que la G6PDH fournit à la fois du NADPH à des systèmes antioxydants et des systèmes pro-oxydants tels que la xanthine oxydase, l'oxyde nitrique synthase et la NADPH oxydase (Hecker & Leopold 2013) qui sont impliqués dans la production de radicaux libres lors d'exercices exhaustifs (Gomez-Cabrera et al. 2005; Gomez-Cabrera et al. 2010; Gomez-Cabrera et al. 2013). Ceci pourrait aussi expliquer l'absence de différence lors de l'exposition à l'hyperoxie.

La surexpression de G6PDH améliore la composition corporelle et les performances physiques

Dans le but de confirmer les données suggérant que la G6PDH serait impliquée dans la régulation de la masse musculaire (Max 1984; Kovacheva et al. 2010), nous avons réalisé une étude de la composition corporelle. Nous avons constaté que la surexpression de G6PDH conduit à une diminution du poids corporel associée à une augmentation de la masse maigre a augmenté et une réduction de de la masse grasse. L'augmentation de masse maigre est dû à une masse musculaire plus importante car la densité minérale osseuse, le poids des organes internes (cœur, foie, reins…) normalisé par le poids corporel étaient similaires entre les souris G6PDHtg et WT alors que le poids des muscles (gastrocnémien, tibialis antérieure, soléaire) normalisés par le poids corporel étaient plus élevés chez les souris G6PDHtg comparés aux souris WT. De plus, l'augmentation du contenue protéique musculaire total en chaine lourde de myosine chez les souris G6PDHtg a confirmé l'augmentation de la masse musculaire chez ces dernières. L'amélioration de la composition corporelle observée chez les souris G6PDHtg était associée à de meilleures performances physiques que celles observées chez les souris WT. En effet, nous avons trouvé que la force musculaire et la consommation maximale d'oxygène étaient plus élevées chez les souris G6PDHtg. L'amélioration de la composition corporelle et notamment l'augmentation de la masse musculaire ainsi que l'augmentation de la force musculaire et de la consommation maximale d'oxygène sont capital dans la sarcopénie,

qui est caractérisé par une diminution de la masse et de la force musculaire (Cruz-Jentoft et al. 2010). En outre, une diminution de la masse grasse aurait également un effet bénéfique sur la fonction musculaire. En effet, au cours de la sarcopénie il y a des infiltrations graisseuses dans le muscle qui sont un facteur prédictif d'invalidité et de mortalité (Visser et al. 2005).

La composition corporelle peut être influencée par l'activité spontanée des animaux et/ou leur comportement alimentaire. Ces deux paramètre ont été évalués chez les souris G6PDHtg et WT et non montré aucune différence entre les deux groupes de souris, ce qui a permis d'écarter ces deux phénomènes pour expliquer les différences de composition corporelle.

Afin d'expliquer, cette masse musculaire plus importante chez les souris G6PDHtg comparées aux WT, nous avons étudié des voies de signalisation

Nos résultats constituent une preuve que la G6PDH joue un rôle central dans la régulation de la masse musculaire comme ceci avait été suggéré par plusieurs études auparavant. En effet, Max (1984) et Kovacheva et al. (2010) ont montré dans plusieurs modèles que l'atrophie musculaire est associée à une diminution de l'activité et du contenu protéique musculaire de la G6PDH diminué et la teneur en protéines tandis que l'hypertrophie musculaire est associée à une augmentation de ces deux paramètres. La régénération musculaire est aussi associée à une activité accrue de la G6PDH (Wagner et al. 1977; Wagner et al. 1978). Pour expliquer cela, il était proposé qu'une augmentation de l'activité de G6PDH conduire à augmenter la synthèse ribose-5-phosphate (R5P) conduisant à une augmentation de la synthèse d'ADN et d'ARN et *in fine* de protéines. Cette hypothèse a été confirmée *in vitro*, où la surexpression de G6PDH a conduit à une augmentation de la vitesse de croissance des cellules en raison d'une synthèse d'ADN plus élevée conduisant à une synthèse de la protéine accrue (Tian et al. 1998; Kuo et al. 2000). Cependant, ceci n'avait jamais été confirmé *in vivo*. Mais, dans notre modèle animal. Nos résultats ont montré un contenue en ADN total dans le muscle gastrocnémien supérieur chez les souris G6PDHtg comparées aux WT, ce qui a confirmé les études mentionnées ci-dessus. De plus, les concentrations plasmatiques en acide urique observées chez les souris G6PDHtg ont attesté que la surexpression de G6PDH augmente le turnover des acides nucléiques. Ceci conférait aux souris G6PDGtg une capacité de synthèse protéique plus importante aux souris G6PDGtg ce qui pourrait augmenter leur synthèse protéique expliquant ainsi l'augmentation de leur masse musculaire. Cependant, ceci est à confirmer par la mesure directe des flux de synthèse protéique. D'autre part, nous avons étudié la voie de signalisation de PI3K/Akt/mTOR afin d'étudier l'impact de la surexpression

de G6PDH sur la traduction des protéines. Nos résultats (activation de Akt et P70S6K) n'ont montré aucune différence entre les souris G6PDHtg et WT.

En résumé, nos travaux ont montré que la surexpression de la G6PDH chez la souris diminue nettement les dommages oxydatifs de l'ADN dans le muscle squelettique. Cependant, les mécanismes à l'origine de cette protection sont étudiés. Étonnamment, nos résultats ont montré une absence de protection de la surexpression G6PDH surexpression contre le stress oxydant induit par des stimuli pro-oxydants surement en raison du fait que le NADPH synthétisé par la G6DPH intervient à la fois dans des systèmes pro et antioxydants. D'autre part, ces travaux sont les premiers à montrer chez la souris que la surexpression de G6PDH améliore la composition corporelle en diminuant la masse adipeuse et en augmentant la masse musculaire surement grâce à une augmentation de la capacité de synthèse protéique via une augmentation de la teneur en ADN au niveau musculaire. L'amélioration de la composition corporelle est associée à une amélioration de la force musculaire et des qualités aérobies.

Finalement, ces travaux montrent que l'amélioration de l'activité de la G6PDH représenterait une bonne stratégie pour améliorer la composition corporelle et la performance physique. Dans un contexte plus large, les effets bénéfiques observés en réponse à la surexpression de G6PDH, conduirait à améliorer la santé de ces souris et finalement augmenterait la longévité comme ceci a été montré chez un modèle de drosophiles surexprimant la G6PDH.

III) Conclusion

L'espérance de vie n'a jamais été aussi longue dans l'histoire de l'humanité. Cependant, ceci conduit à un vieillissement général de la population en général et inévitablement à une augmentation de la prévalence de la sarcopénie, qui à son tour participe à l'augmentation considérable des coûts de soins de santé de nos sociétés. Afin de limiter ce phénomène, élaborer des stratégies efficaces pour prévenir ou traiter la sarcopénie est un défi majeur qui nécessite la compréhension des mécanismes cellulaires et moléculaires impliqués dans son apparition et ceux permettant sa prévention.

Cette thèse a tenté de répondre à trois objectifs généraux. Le premier objectif était de déterminer *in vivo* dans quelle mesure un état redox pro-oxydant dans le tissu musculaire âgé pouvait moduler les voies de signalisation impliquées dans les mécanismes moléculaires de la sarcopénie. Le deuxième objectif était de montrer que le retour à un fonctionnement normal de ces voies de signalisation nécessite une restauration de l'homéostasie redox. Enfin, le troisième objectif de cette thèse était d'identifier de possibles acteurs et mécanismes moléculaires permettant le maintien et/ou la restauration de l'homéostasie rédox.

Dans une première étude réalisée chez les rats âgés, nous avons constaté que le stress oxydant lié à l'âge conduit à une altération de la voie PI3K/Akt/mTOR suggérant une diminution de la synthèse des protéines alors que dans le même temps une augmentation de l'expression de MuRF1 et MAFbx suggérait une augmentation de la protéolyse par le système ubiquitine-protéasome. En outre, une diminution de la fonction mitochondriale et la genèse a été trouvé. Dans un second temps, nous avons montré qu'une thérapie de remplacement à l'hormone de croissance chez des rats âgés permet de prévenir la sarcopénie grâce à des effets antioxydants, anaboliques et anti cataboliques. Dans cette première étude, il est apparu que la G6PDH pouvait être un possible candidat expliquant l'effet antioxydant de l'hormone de croissance.

Dans les études 2 et 3, nous avons constaté que des souris transgéniques surexprimant la G6PDH ont une amélioration de la composition corporelle qui se caractérise par un poids corporel plus faible, une diminution de la masse grasse et une augmentation de la masse musculaire. En outre, nous avons observé que les souris G6PDHtg présentent une amélioration des qualités aérobies et de la force musculaire. De plus, nous avons montré une diminution des dommages oxydatifs de l'ADN chez les souris G6PDHtg. Bien que, de façon surprenante, nous n'avons pas trouvé d'effet protecteur de la surexpression contre le stress

oxydant induit par l'exercice exhaustif, un effet délétère était tout aussi absent.

Bien que les mécanismes permettant d'expliquer les effets bénéfiques de la surexpression de G6DPH soient encore à préciser, nos résultats ouvrent une porte vers le développement de stratégies pour lutter contre la sarcopénie mais de façon plus générale pour améliorer la composition corporelle et la performance physique axées sur une activation de la G6PDH. Toutefois, avant de développer de telles stratégies, d'autres études sont nécessaires afin de s'assurer de l'absence de danger de l'activation de la G6DPH.

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Abstract	Résumé
Aging is characterized by a decrease in muscle mass and strength causing a deterioration of physical performance, called sarcopenia. Muscle atrophy can be explained by a negative protein turnover, impaired mitochondrial dynamics, a decreased muscle regeneration capacity and myonuclei apoptosis. A decreased production of anabolic hormones and a chronic oxidative stress (OS) which leads to excessive oxidative damage would be involved in these alterations. Physical exercise and hormone replacement therapies are effective to combat sarcopenia. The restoration of a redox homeostasis may play a central role in their beneficial effects and would involve an up-regulation of the glucose-6-phosphate dehydrogenase enzyme. The main objectives of this thesis were to determine *in vivo* to what extent a pro-oxidant redox status in aged muscle may modulate signaling pathways involved in sarcopenia, and to investigate whether return to their normal functioning requires a restoration of the redox homeostasis. The third objective was to identify actors and their possible cellular mechanisms in the maintenance and/or the restoration of the redox status. In a first study in old rats, we first confirmed that sarcopenia is associated with OS. In a second time, we found that a growth hormone replacement therapy in olds rats prevents sarcopenia by acting as a double-edged sword, antioxidant as well as myogenic, associated with an up-regulation of G6DPH. In a second study, we found that transgenic mice overexpressing G6PDH showed improved body composition and physical performances associated. In a third study, we found that overexpression of G6DPH improves DNA oxidative damage in resting condition. However, the expected protective effect of G6PDH overexpression against oxidative stress induced by pro-oxidizing stimuli was not present.	Le vieillissement est caractérisé par une diminution de la masse et la force musculaire entraînant une détérioration des performances physiques, appelée sarcopénie. L'atrophie musculaire peut être expliquée par un turnover protéique négatif, une détérioration des dynamiques mitochondriales, une diminution de la capacité de régénération du muscle ainsi que par l'apoptose des noyaux musculaires. La diminution de la sécrétion d'hormones anabolisantes et un stress oxydant (OS) chronique conduisant à des dommages oxydatifs excessifs, seraient impliqués dans ces modifications. L'Exercice physique et les thérapies de remplacement hormonales sont efficaces pour lutter contre la sarcopénie. Une restauration de l'homéostasie redox pourrait avoir un rôle central dans la lutte contre la sarcopénie et impliquerait une activation de la glucose-6-phosphate déshydrogénase. Les principaux objectifs de cette thèse étaient de déterminer *in vivo,* si un SO chronique dans le muscle âgé altère les voies de signalisation impliquées dans la sarcopénie, et de chercher si le retour à un fonctionnement normal de ces voies nécessite une restauration de l'homéostasie redox. Certains paramètres et leurs mécanismes pouvant intervenir sur le maintien ou la restauration du SO ont été recherchés. Dans une première, nous avons confirmé que la sarcopénie est associée au OS chez le rat. Puis nous avons constaté qu'un traitement à l'hormone de croissance chez le rat peut prévenir la sarcopénie via un effet antioxydant et myogénique, associé à une activation de la G6DPH. Une seconde étude a monté des souris transgéniques surexprimant G6PDH présentaient une amélioration de la composition corporelle et des performances physiques. Une dernière étude a montré que la surexpression de G6DPH diminuait les dommages oxydatifs de l'ADN au repos. De façon surprenante, la surexpression de la G6PDH n'a pas d'effet protecteur vis à vis du SO induit par les divers stimuli pro-oxydants.

Keywords: sarcopenia, oxidative stress, exercise, growth hormone, skeletal muscle, G6DPH	**Mots-clés:** sarcopénie, stress oxydant, exercice, hormone de croissance, muscle, G6DPH

Laboratoire « Mouvement, Sport, Santé »

Avenue Robert Schuman 35170 Bruz

Tél : + 33(2) 90 09 15 80